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Methanolysis of Nitrocefin Catalyzed by One and Two Zn^{2+} Ions. A Simplified Model for Class B β-Lactamases

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The methanolysis of nitrocefin (**1**) was investigated at 25 °C in anhydrous methanol as a function of $[Zn^{2+}]$ and $^{S}_{S}$pH as a model for the chemistry believed to occur in the active site of Zn^{2+}–β-lactamases. In the absence of metal ion, the $^{S}_{S}$pH vs rate constant profile shows a long plateau region having $k_{obs} = 9 \times 10^{-5}$ s^{-1} between $^{S}_{S}$pH 7.5 and 12 generated after ionization of the COOH of **1** (kinetic $^{S}_{S}pK_a$ of 7.34) followed by attack of CH_3O^- with $k_{CH_3O} = 1.18$ M^{-1} s^{-1}. Strong catalysis of the methanolysis is observed at all $^{S}_{S}$pH values between 7.95 and 11.34 in the presence of as little as 0.05–3 mM Zn^{2+}. Plots of the pseudo-first-order rate constant for methanolysis (k_{obs}) as a function of $^{S}_{S}$pH reveal a saturation phenomenon indicative of formation of a **1**:Zn^{2+} complex, followed by a linear section indicative of the intervention of a second Zn^{2+} ion in promoting the methanolysis of the complex. The two processes can be separated since the slope of the linear part of the plots gives the second-order rate constant (k_2^{obs}) for the second Zn process, while the intercept gives the spontaneous rate constant for methanolysis of **1**:Zn^{2+} ($k_{cat.}^{obs}$). A plot of log $k_{cat.}^{obs}$ vs $^{S}_{S}$pH is bell-shaped, maximizing at $\sim^{S}_{S}$pH 10, with ascending and descending domains both first order in $[CH_3O^-]$. A plot of log k_2 vs $^{S}_{S}$pH gives an ascending domain second order in $[CH_3O^-]$, followed by a plateau above $^{S}_{S}$pH 9.5. The data are analyzed in terms of a one-Zn and two-Zn model in which the former involves rate-limiting attack of methoxide on a **1**:Zn^{2+} complex up to the $^{S}_{S}$pH maximum and a spontaneous reaction of **1**:Zn^{2+}:$(CH_3O^-)_2$, while the latter involves bimolecular attack of $Zn^{2+}(CH_3O^-)_2$ on both **1**:Zn^{2+} and **1**:Zn^{2+}:$(CH_3O^-)_2$. The relevance of these observations is discussed in terms of the currently accepted mechanism for hydrolyses of β-lactams promoted by the Zn^{2+}–β-lactamases.

Introduction

The β-lactam antibiotics, comprising the penicillin, cephalosporin, and carbapenem families, are widely used to combat bacterial infections.[1] Bacterial resistance to these antibiotics[2] through the evolution of β-lactamases presents a serious threat to human health. Although the mechanism of action of the class A, C, and D serine β-lactamases has been well-documented, and there are presently available useful inhibitors of these enzymes,[3] the recently discovered class B Zn^{2+}–β-lactamases[4] hydrolyze a broad spectrum of β-lactam antibiotics but have no useful inhibitors. Crystal structures are available for several members of the Zn^{2+}–β-lactamases[4] and show the active sites to contain two Zn^{2+} ions bound to various peptide ligands and a coordinated HO^- which either bridges the two Zn^{2+} ions or is singly coordinated. By analogy with other metallohydrolases such as carboxypeptidase, angiotensin converting enzyme, and carbonic anhydrase, the Zn^{2+}-coordinated HO^- is thought to be the active nucleophile that attacks the C=O unit of the β-lactam.[5]

Despite the fact that several members of the mononuclear[6] and dinuclear[7] Zn^{2+}–β-lactamases are under active investigation, a detailed picture of the mechanism, and particularly

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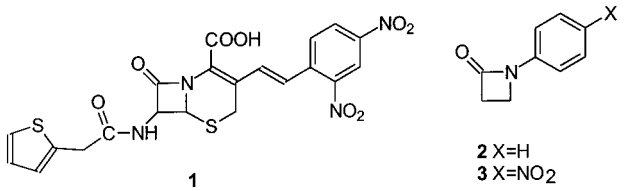
the role of the second Zn^{2+} in the mechanism of enzyme-promoted hydrolysis of β-lactams, has yet to emerge. A recent suggestion for the mechanism operative in the dinuclear enzyme *B. fragilis*[7] indicates that one Zn^{2+} acts to bind the COO^- of the substrate (required for binding all good substrates[8]) and possibly with the lactam N, thus enhancing its leaving ability as an anionic $^-N-Zn^{2+}$ form. There is, however, uncertainty with respect to the universality of COO^- binding to the metal since a crystal structure available for an IMP-1 metallo-β-lactamase from a *Pseudomonas aeruginosa* enzyme:inhibitor complex indicates the COO^- of the inhibitor interacts with lysine-161.[4c] Furthermore, modeling of the interaction of substrate interactions with the active site of the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* suggests that the substrate COO^- binds to serine-187,[4d] while a second modeling study[4e] with a new crystal form of the β-lactamase from *B. cereus* that contains two Zn^{2+} ions in the active site, indicates the substrate COO^- displaces a water on one Zn^{2+} ion, with possible interaction to maintain pentacoordination at the metal. In the latter study it was suggested that the *B. cereus* enzyme might be an evolutionary intermediate between the mono- and dinuclear enzymes in that it can function efficiently with one metal ion as the active nucleophile but will accommodate a second. The role of the histidine-coordinated, but nonsubstrate, binding Zn^{2+} is to provide the nucleophilic metal-bound HO^-. Such an arrangement must be particularly effective in promoting the nucleophilic attack and breakdown of the tetrahedral intermediate, since the rate-limiting step for the *B. fragilis* enzymatic cleavage of β-lactams is protonation of the $^-N-Zn^{2+}$ following the breakdown of the tetrahedral intermediate. On the other hand, for the earlier studied, and apparently mono-nuclear β-lactamase from *B. cereus*, the rate-limiting step is cleavage of the tetrahedral intermediate,[6] formed from rapid attack of the sole active site $Zn^{2+}-HO^-$. It has been suggested[7a] that the two-metal ion mechanism is similar to that proposed for other dinuclear hydrolases[9] wherein one metal ion provides the bound HO^- nucleophile and serves as a counterpart for the oxyanion hole in stabilizing the tetrahedral intermediate, while the other helps to (a) position the substrate for proper nucleophilic attack, (b) polarize further the (N)C=O scissile bond, and (c) stabilize the (−)-charge developed on the leaving group.

There is some early work investigating the effects of divalent metal ions on the aqueous hydrolysis of benzyl penicillin[10a−c] and cephaloridine[10c] that shows very marked M^{2+}-catalysis. From the saturation appearance of the k_{obs} vs $[M^{2+}]$ plots, the metal ion appears to bind to the carboxylate of the penicillin or cephalosporin, promoting the attack of external hydroxide on the β-lactam C=O, with possible binding to the lactam N facilitating breakdown of the tetrahedral intermediate. More recently β-lactam degradation in methanol has been reported[11] to be promoted by Cd^{2+} under non-pH controlled conditions, although a detailed mechanism for activity was not reported. Importantly, in none of the above studies was evidence presented indicative of the involvement of more than one metal ion in the hydrolysis or methanolysis. In some cases, modeling[12] the chemistry believed to occur in the active sites of dinuclear enzymes[13] has provided interesting small molecule analogues displaying cooperative effects in promoting acyl or phosphoryl transfer processes. A few simple mononuclear and dinuclear models for the dinuclear $Zn^{2+}-\beta$-lactamases have been presented,[14] but the catalysis of hydrolysis of the usual mechanistic probe β-lactam, nitrocefin (**1**), was reported to be modest. Moreover, a catalytic role for the second Zn^{2+} could not be established since mononuclear complexes seemed to be as effective as dinuclear complexes.



Previously we described[15] the catalysis of methanolysis of acetylimidazole promoted by Zn^{2+} and Co^{2+} in buffered methanol. In contrast to the situation in water where the formation of metal hydroxide gels and precipitates often seriously complicates mechanistic studies of M^{2+}-catalyzed acyl transfer reactions, Zn^{2+} is soluble in MeOH throughout the entire ^s_spH region surrounding ionization of $Zn^{2+}(CH_3OH)_x$. Due to its reduced dielectric constant relative to water (31.5 vs 78.5 at 25 °C)[16] methanol may serve as an interesting host solvent that might be relevant to the local dielectric constant of the enzyme interior,[17] and may prove particularly effective for studying metal ion promoted acyl [15,18] and phosphoryl transfer[19] processes. Moreover, the reduced

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dielectric constant may have another benefit in promoting the association of metal ions with anionic substrates thus leading to stronger M^{2+}:substrate interactions than are displayed in water. Herein we report studies of the methanolysis of nitrocefin (**1**) promoted by $Zn^{2+}(ClO_4^-)_2$ in buffered methanol for which the evidence clearly points to two concurrent acyl transfer mechanisms involving, respectively, one and two Zn^{2+} ions. The processes involve the intermediacy of a Zn^{2+}:**1** complex, undoubtedly bound through the **1**−COO^- since neither of β-lactam **2** nor **3** is subject to catalysis by added Zn^{2+}.[20]

Experimental Section

Materials. Nitrocefin was purchased from Oxoid. Methanol (99.8% anhydrous), sodium methoxide (0.5 M), $HClO_4$ (70%, BDH), and acetonitrile (99.8% anhydrous) were all purchased from Aldrich and used without any further purification. $Zn(ClO_4)_2 \cdot 6H_2O$ was purchased from Aldrich and dried by heating under vacuum to form $Zn(ClO_4)_2 \cdot H_2O$ which was used for the kinetics. $Zn(TfO)_2$ was purchased from Aldrich and used as supplied for the titrations. Lactams **2** and **3** were prepared according to published procedures.[21]

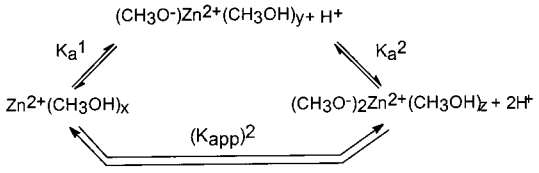
^s_spH Measurements. The $CH_3OH_2^+$ concentration was determined using a Radiometer pHC4000-8 combination (glass/calomel) and a Radiometer pHC2201 double junction electrode for kinetic and titration measurements, respectively, calibrated with Fisher Certified Standard aqueous buffers (pH = 4.00 and 10.00). A 1.0 M solution of $LiClO_4$ was used in the second junction of the electrode. Values of ^s_spH were calculated by adding a correction constant of 2.24 to the experimental meter reading (^s_wpH). This method was first described by Bates[22] for a molality scale (correction constant 2.34), and later by Bosch et al.[23] for a molar correction constant of 2.24 units. The $^s_s pK_a$ values of buffers used for the present kinetic studies were obtained from the literature[23] or measured at half-neutralization of the bases with 70% $HClO_4$ in MeOH.

$^s_s pK_a$ Determination. The potentiometric titrations of the Zn salt and acetic acid in methanol were performed using a Radiometer Vit 90 Autotitrator under anaerobic conditions (Ar) at ambient temperature. The concentrations of metal salt and sodium methoxide titrant (prepared from stock 0.5 M methoxide in a Sure Seal bottle) were 2.00×10^{-3} and $(1.88-2.12) \times 10^{-2}$ M, respectively. The latter was calibrated by titrating standardized HCl in water, with the endpoint taken to be ^w_wpH = 7. The ionic strength of the solutions was kept at 0.01 M with tetrabutylammonium perchlorate. Obtaining reproducible titration curves proved to be problematic unless a standard protocol was adopted. After each titration the electrode was immersed in pH 4.00 aqueous buffer until the reading stabilized at pH 4.00, a process that takes between 10 and 20 min. The electrode was rinsed with MeOH, dried with a tissue, and then used for the next titration. The values of the acid dissociation constants in methanol ($^s_s pK_a$) were calculated using the computer program PKAS.[24] The $^w_w pK_w$ in the program was replaced by the autoprotolysis constant for methanol at 25 °C ($^s_s pK_{MeOH}$ = 16.77).

Kinetic Measurements. The molar extinction coefficients of nitrocefin and its methanolyzed product, in methanol as determined in this work, are ϵ_{390} = 14 200 and 4020 M^{-1} cm^{-1} and ϵ_{490} = 680 and 15 400 M^{-1} cm^{-1}, respectively. The rate of appearance of the methanolyzed product was followed by monitoring the increase in absorbance of buffered methanol solutions at 480 nm, with an OLIS modified Cary 17 UV−vis spectrophotometer or an Applied Photophysics SX-17MV stopped-flow reaction analyzer at 25.0 ± 0.1 °C. All runs were at least repeated in duplicate. Reactions were monitored under pseudo-first-order conditions of excess Zn^{2+} ions, in the range of $(0.05-4) \times 10^{-3}$ M. In the case of slow reactions using conventional UV analysis, these were initiated by addition of an aliquot of a 2.0×10^{-3} M stock solution of **1** in CH_3CN (with 2.5% MeOH) to 2.5 mL of the buffered reaction mixture (final concentration of **1** is $(0.8-2) \times 10^{-5}$ M). For reactions followed using the stopped-flow apparatus, one drive syringe was charged with **1** in 0.02 M buffer, while the second syringe contained twice the desired concentration of Zn^{2+} and 0.02 M buffer. Reactions were followed for at least four half-lives and displayed good first-order behavior. Pseudo-first-order rate constants (k_{obs}) for methanolysis were determined by NLLSQ fitting of the absorbance vs time traces to a standard exponential model. *N*-methylimidazole ($^s_s pK_a$ = 7.60), *N*-ethylmorpholine ($^s_s pK_a$ = 8.28), trimethylamine ($^s_s pK_a$ = 9.80 [23b]), triethylamine ($^s_s pK_a$ = 10.78 [23b]), and diisopropylamine ($^s_s pK_a$ = 11.45 [23b]) buffers, partially neutralized with $HClO_4$, were used to control the ^s_spH. Total buffer concentration was 2×10^{-2} M. ^s_spH measurements were performed before and after each experiment. To avoid any chloride ion contamination from the glass electrode that might affect the metal ion reactions, duplicate solutions were prepared: one for ^s_spH measurements and the second portion being used for kinetics. In all cases, ^s_spH values measured before and after reaction were consistent to within 0.05 units.

Results

(a) Potentiometric Titration of Zn^{2+}. Shown in Figure 1 is a titration profile for a methanol solution containing 0.01 M N(*n*-Bu)$_4ClO_4$ and 2×10^{-3} M $Zn^{2+}(TfO^-)_2$ in the absence and presence of equimolar acetic acid. As we have noted previously,[15b] the steepness for the titration profile for Zn^{2+} alone, and the fact that there are two methoxides consumed, indicates a cooperative binding of CH_3O^- to Zn^{2+} as in eq 1a



(1a)

where the second dissociation constant is larger than the first. Analysis of the profile[24] gives values of 10.34 ± 0.14 and

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(20) Montoya-Pelaez, P.; Brown, R. S. Unpublished results. While we see no propensity of Zn^{2+} to promote the methanolysis of simple lactams **2** and **3**, we have conducted preliminary experiments monitoring the methanolysis of 0.25 M penicillin G by ^{1}H NMR in CD_3OD at ^s_spH 9.69 in the presence of 2.5×10^{-3} M $Zn(OTf)_2$ (1%). This process exhibits good first-order kinetics, the k_{obs} being 2.3×10^{-4} s^{-1}, a catalysis of some 180-fold over the background reaction at ^s_spH 10.7 ($k_{obs} = 1.25 \times 10^{-6}$ s^{-1}).
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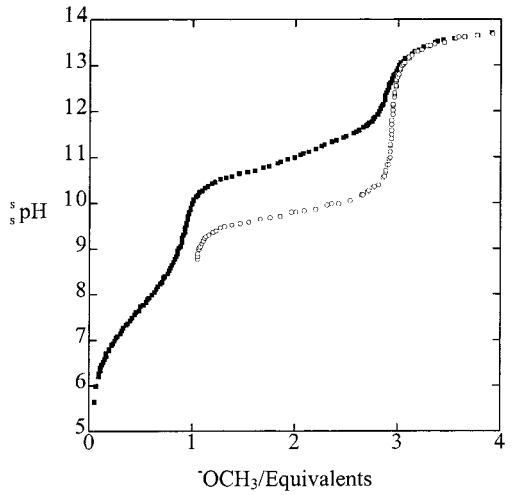
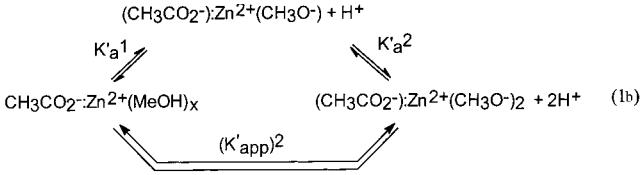


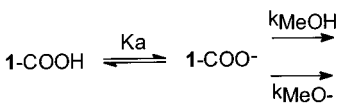
Figure 1. Titration profiles for 2×10^{-3} M $Zn(ClO_4)_2$ in methanol in the absence (○) and presence (■) of equimolar acetic acid and 0.01 M N(*n*-Bu)$_4ClO_4$. Acetic acid profile is offset by 1 equiv of CH_3O^- relative to the profile for Zn^{2+} alone to facilitate visual comparison.

9.25 ± 0.18 for pK_a^1 and pK_a^2, respectively ($^{s}_{s}$pK_{app} 9.79 ± 0.16 for the midpoint of titration). In the presence of equimolar acetic acid, the titration profile shows evidence of three events: an ionization at $^{s}_{s}$pK_a = 7.70 ± 0.19 corresponding to the ionization of acetic acid lowered from the value of 9.37 ± 0.14 we obtained in the presence of 0.01 M N(*n*-Bu)$_4ClO_4$ (9.71 ± 0.22 in MeOH without N(*n*-Bu)$_4ClO_4$, lit. 9.63[23a]) in the absence of metal ion and two other ionizations having $^{s}_{s}$pK_a values of 10.94 ± 0.02 and 11.08 ± 0.25 corresponding to association of CH_3O^- to the $Zn^{2+}{}^{-}O_2CC\ H_3$ (midpoint of the titration, $^{s}_{s}$pK'_{app} 11.01). Shown in eq 1b is a process for titration of $(CH_3OH)_xZn^{2+}$:$^{-}O_2CCH_3$, analogous to that shown in eq 1a for Zn^{2+} alone.



(1b)

(b) Kinetics. Given in Table 1S, Supporting Information, are the k_{obs} vs $^{s}_{s}$pH data for the methanolysis of **1** in the absence of metal ion extrapolated to [buffer] = 0 at μ = 0. The complete $^{s}_{s}$pH vs log k_{obs} profile shown in Figure 2 consists of three domains that can be analyzed in terms of the mechanism presented in eq 2 where P is the methanolysis product. The ascending portion at low $^{s}_{s}$pH with a kinetic $^{s}_{s}$pK_a of 7.34 for the COOH, gives rise to a plateau from 7.5 to 12 (k_{MeOH} = 8.96×10^{-5} s^{-1}), followed by a CH_3O^- domain having k_{MeO} = 1.18 M^{-1} s^{-1}.



(2)

In the presence of Zn^{2+}, marked catalysis of the methanolysis of **1** is observed. There are two general observations

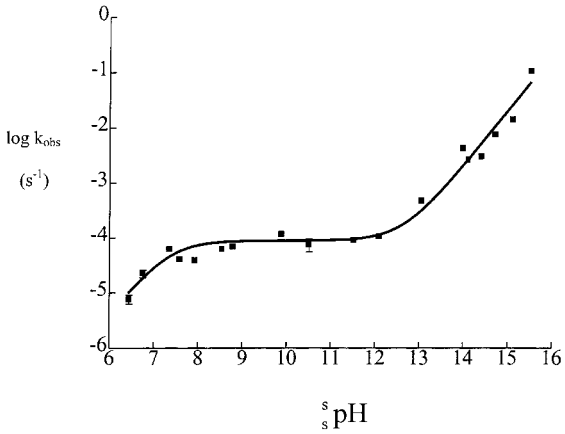


Figure 2. Plot of the log k_{obs} vs $^{s}_{s}$pH for methanolysis of nitrocefin (**1**), T = 25 °C.

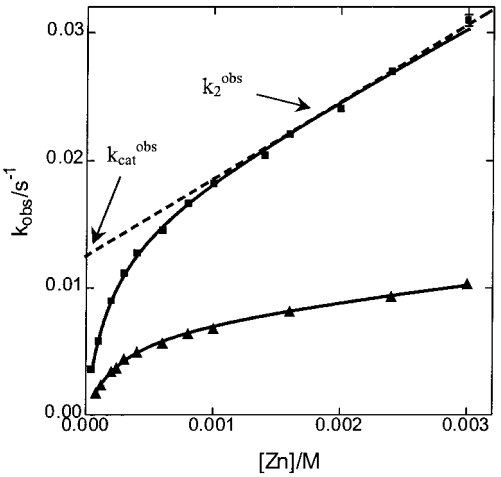
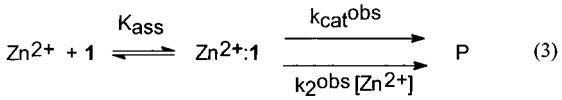


Figure 3. Plots of pseudo-first-order rate constants for the methanolysis of nitrocefin (**1**) vs $Zn(ClO_4)_2$ at 25 °C; $^{s}_{s}$pH = 8.55 (▲) and $^{s}_{s}$pH = 8.91 (■).

revealed in the k_{obs} vs [Zn^{2+}] data given in Tables 2S−12S, Supporting Information, that must be explained. First, at a given $^{s}_{s}$pH, plots of k_{obs} vs [Zn^{2+}] are curved, suggestive of a saturation Zn^{2+} + **1** ↔ **1**:Zn^{2+} phenomenon. However, as revealed by the two representative plots at $^{s}_{s}$pH 8.55 and 8.91 given in Figure 3, these do not plateau, but rather yield a linear portion at high [Zn^{2+}]. Notably, none of these plots shows the characteristic upward curvature which would suggest the involvement of catalytically active Zn^{2+} dimers. Since neither the methanolysis of lactam **2** nor **3** shows any catalysis by Zn^{2+}, the saturation phenomenon seen with **1** must be attributable to association of its carboxylate with the metal ion.[20] The linear portion of the plot, occurring at a [Zn^{2+}] greater than required for saturation, must then arise from a second Zn^{2+} interacting with the Zn^{2+}:**1** complex as schematized in eq 3.



(3)

$$k_{obs} = \frac{(K_{ass}[Zn^{2+}])(k_2^{obs}[Zn^{2+}] + k_{cat}^{obs})}{(1 + K_{ass}[Zn^{2+}])} \quad (4)$$

Table 1. $k_{cat.}^{obs}$, k_2^{obs}, and K_{assoc} Constants for the Zn^{2+}-Promoted Methanolysis of Nitrocefin (**1**) at Various ^s_spH Values, $T = 25$ °C, $I = 0.02$

^s_spH	$K_{cat.}^{obs}$ (s^{-1}) × 10^3	k_2^{obs} (M^{-1} s^{-1})	K_{assoc} (M^{-1}) × 10^3 [a]
7.95	0.99 ± 0.3	0.253 ± 0.075	4.8 ± 0.1
8.26	2.3 ± 0.2	0.367 ± 0.040	6.2 ± 0.1
8.55	5.7 ± 0.4	1.53 ± 0.15	6.7 ± 0.1
8.91	11.6± 0.3	6.39 ± 0.14	10 ± 1
9.49	50.2 ± 3	15.6 ± 1.1	3.1 ± 0.1
9.66	68.7 ± 1.5	14.8 ± 1.5	7.7 ± 0.2
9.88	78.3 ± 2	16.2± 1.1	12 ± 1
10.01	78.4 ± 0.7	14.2± 2.3	18 ± 9
10.30	52.0 ± 0.1	24.7 ± 4.7	*b*
10.95	22.1 ± 0.6	18.0 ± 0.6	*b*
11.34	15.5 ± 0.7	15.7 ± 0.7	*b*

[a] K_{assoc} defined as the association constant of **1**:Zn^{2+} as in eqs 3 and 4. [b] Value too large to be determined from data since plots of k_{obs} vs $[Zn^{2+}]$ are linear commencing at the lowest concentrations (5×10^{-5} M).

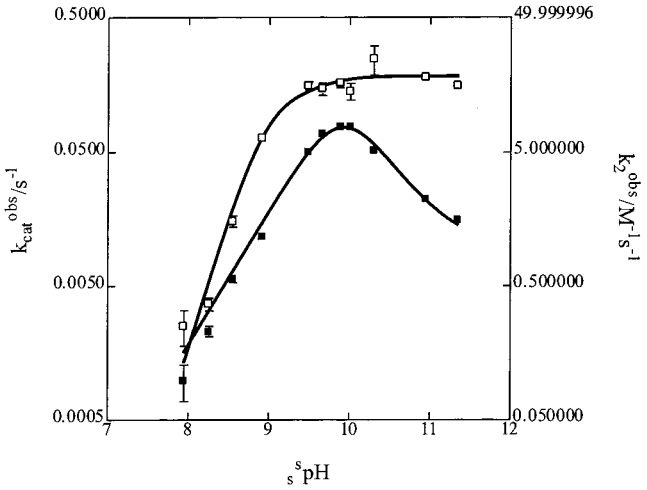


Figure 4. Plot of log $k_{cat.}^{obs}$ (■) and log k_2^{obs} (□) vs ^s_spH for $Zn(ClO_4)_2$ catalyzed methanolysis of nitrocefin (**1**), $T = 25$ °C. Individual data points determined as described in text. Lines through the data computed from fits to eqs 6 and 7 respectively.

The various rate and equilibrium constants described in eq 3 can be evaluated by fitting the k_{obs} vs $[Zn^{2+}]$ data at all ^s_spH values to eq 4. However, evaluation of the $k_{cat.}^{obs}$ and k_2^{obs} rate constants between ^s_spH 7.95 and 10.95 is greatly simplified by considering that the slopes of the linear parts of the k_{obs} vs $[Zn^{2+}]$ plots give the k_2^{obs} constants at each ^s_spH, while the intercepts give the $k_{cat.}^{obs}$ terms. These values are given in Table 1, along with the K_{assoc} terms for formation of the Zn^{2+}:**1** complex, which were determined from fitting the k_{obs} vs $[Zn^{2+}]$ data at each ^s_spH value to eq 4, setting the $k_{cat.}^{obs}$ and k_2^{obs} to values determined from the slopes and intercepts of the plots as constants. Notable are the K_{assoc} data for formation of **1**:Zn^{2+}, suggesting that binding becomes stronger with increasing ^s_spH (vide infra).

Shown in Figure 4 are the plots of log $k_{cat.}^{obs}$ and log k_2^{obs} vs ^s_spH that provide information concerning the kinetic dependence of these rate constants on $[CH_3O^-]$. The $k_{cat.}^{obs}$ plot shows a bell-shaped ^s_spH dependence of the reaction of the Zn^{2+}:**1** complex ($k_{cat.}^{obs}$), maximizing at $\sim^s_s$pH 10, with ascending and descending domains each first order in $[CH_3O^-]$ with a hint of a developing plateau at ^s_spH > 11. The ^s_spH dependence of the reaction involving the second Zn^{2+} (k_2^{obs}), gives an ascending domain second order in $[CH_3O^-]$, followed by a plateau at ^s_spH > 9.5.

Discussion

(a) Titration. A curious feature of the titration of Zn^{2+} ions in methanol, aside from the solutions being homogeneous throughout the ^s_spH region surrounding ionization of the $Zn^{2+}(CH_3OH)_x$ complex, is the cooperativity seen for formation of $Zn^{2+}(CH_3O^-)_2$. Thus, in the absence of additional ligands, two methoxides are consumed between $\sim^s_s$pH 8.5 and 10.5 (apparent $^s_s pK_{app}$ of 9.8 for midpoint of the titration)[15b] which on analysis[24] yields two $^s_s pK_a$s of 10.34 ± 0.14 and 9.25 ± 0.18 for ionization of the first and second $Zn^{2+}(HOCH_3)$. Our work appears to be the first describing this phenomenon for association of CH_3O^- to metal ions such as Zn^{2+}, although it is known that zinc halides (X = Cl^-, Br^-, and I^-) show preference for the formation of the bis-halide compared to the mono-halide in dimethyl sulfoxide (DMSO)[25] and methanol.[26] For example,

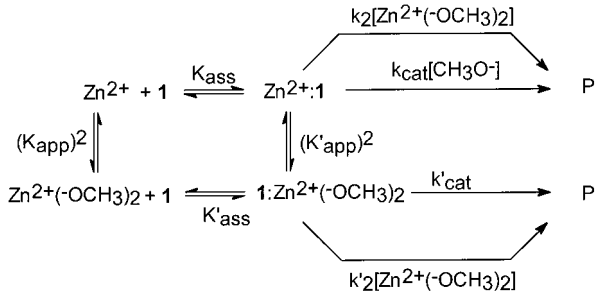
$$(CH_3OH)_nM^{2+} + 2Cl^- \overset{K_1}{\rightleftharpoons} (CH_3OH)_xM^{2+}(Cl^-) + Cl^- \overset{K_2}{\rightleftharpoons} (CH_3OH)_yM^{2+}(Cl^-)_2 \quad (5)$$

Doe et al.[26a] determined K_1 and K_2 stability constants for Zn−Cl in methanol of (7.76 ± 0.18) × 10^3 and (1.74 ± 0.10) × 10^4 M^{-1} (eq 5). They have attributed the cooperativity to a change in the Zn^{2+}-complex structure accompanying the binding of the first Cl^- giving a difference in charge density on Zn^{2+} as it transforms from an octahedral structure ($Zn^{2+}(MeOH)_6$) to a tetrahedral one, $ZnX_2(MeOH)_2$, and also to the entropy effect from the decrease in the number of coordinating MeOH molecules substituted by Cl^-. A similar effect could be operative in the case of methoxide binding to Zn^{2+}.

Titration of 2 × 10^{-3} M $Zn^{2+}(ClO_4^-)_2$ in methanol containing 2 × 10^{-3} M acetic acid reveals an ionization of $^s_s pK_a$ of 7.70 corresponding to that of CH_3COOH producing a Zn^{2+}-bound acetate, followed by the consumption of two more methoxides in what appear to be sequential, and noncooperative, ionizations having $^s_s pK_a^2$ and $^s_s pK_a^3$ values of 10.94 and 11.08 (midpoint of the titration ∼ 11). While it might be contended that the second or third ionizations (of CH_3COO^-:$Zn^{2+}(CH_3OH)_{1,2}$) serve to displace the bound acetate, the two titration profiles shown in Figure 1 do not become superimposable until at least ^s_spH 12.5, requiring that the species formed after the third ionization must be $(CH_3O^-)_2$:Zn^{2+}:$^-OOCCH_3$. The fact that the ionization of the acetic acid in the absence of Zn^{2+} has a $^s_s pK_a$ value of 9.37 (this work, in 0.01 M N(*n*-Bu)$_4ClO_4$) indicates that the association constant (K_{assoc}) of $Zn^{2+}(^-O_2CCH_3)$ must be ∼5 × 10^4 M^{-1}. By analogy, binding should be observed with **1** as well[27] and at high ^s_spH, a $(CH_3O^-)_2$:Zn^{2+}:**1** complex is expected which we show below to be kinetically important.

(25) (a) Ahrland, S.; Björk, N.-O. *Acta Chem. Scand. A* **1976**, *30*, 265. (b) Ahrland, S.; Björk, N.-O.; Portanova, R. *Acta Chem. Scand. A* **1976**, *30*, 270.
(26) (a) Doe, H.; Kitagawa, T. *Inorg. Chem.* **1982**, *21*, 2272. (b) Doe, H.; Shibagaki, A.; Kitagawa, T. *Inorg. Chem.* **1983**, *22*, 1639.

Scheme 1



(b) Kinetics. (i) Spontaneous Methanolysis. In the absence of Zn^{2+} the $^{s}_{s}$pH vs log k_{obs} profile for methanolysis of **1** shown in Figure 2 suggests the kinetic processes shown in eq 2. Throughout the long plateau region in the figure between $^{s}_{s}$pH 7.5 and 12, the background reaction likely stems from an efficient general base assisted delivery of MeOH promoted by the **1**−COO^{-}, generated after the ionization of **1**:COOH (kinetic $^{s}_{s}pK_a = 7.34 \pm 0.09$). Since all the Zn^{2+}-promoted reactions described subsequently were conducted within this $^{s}_{s}$pH region, the spontaneous rate constant ($k_{MeOH} = (8.96 \pm 0.91) \times 10^{-5}$ s^{-1}, $t_{1/2} = 129$ min) can be used as a background to which the effectiveness of Zn^{2+} catalysis can be compared. At higher $^{s}_{s}$pH, direct nucleophilic attack of CH_3O^{-} on the β-lactam occurs with a rate constant of 1.18 ± 0.28 M^{-1} s^{-1}. In no case is there observed the buildup of intermediates during the reaction which indicates nucleophilic attack or breakdown of a reversibly formed tetrahedral intermediate is rate-limiting.

(ii) Zn^{2+}-Promoted Methanolysis. Zn^{2+} catalyzed methanolysis of **1** is particularly rich in information as evidenced by the kinetic dependence on both [Zn^{2+}] and $^{s}_{s}$pH which reveals three important observations that need to be explained by any mechanism. These are (1) the observation of clear saturation behavior in the plots of k_{obs} vs [Zn^{2+}] (see Figure 3) followed by a linear section attributable to the involvement of a second Zn^{2+} reacting with a Zn^{2+}:**1** complex, (2) a first order in [CH_3O^{-}] increase and first-order in [CH_3O^{-}] decrease in the log $k_{cat.}^{obs}$ vs $^{s}_{s}$pH profile (Figure 4) followed by a hint of a plateau at $^{s}_{s}$pH > 11, and (3) a second order in [CH_3O^{-}] dependence of the log k_2^{obs} vs $^{s}_{s}$pH profile (also in Figure 4) followed by a plateau above $^{s}_{s}$pH 9.5. Given in Scheme 1 is a set of processes consistent with the kinetics which is an expanded variant of eq 3. In the scheme are kinetically important species which are supported by the Zn^{2+} and acetic acid titration data, namely, the formation of $Zn^{2+}(CH_3O^{-})_2$ from Zn^{2+} at $\sim^{s}_{s}$pH of 9.7, and the formation of $(CH_3O^{-})_2Zn^{2+}$:**1** from Zn^{2+}:**1**. Based on that scheme, the expressions given in eqs 6 and 7 can be derived to describe the $^{s}_{s}$pH behavior of $k_{cat.}^{obs}$ and k_2^{obs} where $^{s}_{s}K_{app}$ and $^{s}_{s}K'_{app}$ refer to the dissociation constants for the $Zn^{2+}(CH_3OH)_x$ and $(CH_3OH)_2Zn^{2+}$:**1** complexes and $^{s}_{s}K_{MeOH}$ refers to the autoprotolysis constant of MeOH ($10^{-16.77}$).[23] NLLSQ fitting of each set of data to the appropriate expression, setting K_{app} at the constant value of $10^{-9.8}$ based on the $Zn^{2+}(CH_3OH)_x$ titration results, generates the lines through the data shown in Figure 4, the computed constants being given in Table 2.

Table 2. Computed Rate and Dissociation Constants for Zn^{2+} Promoted Methanolysis of Nitrocefin (**1**) from Independent Fits of $k_{cat.}^{obs}$ and k_2^{obs} vs $^{s}_{s}$pH to Equations 6 and 7

	k_x (M^{-1} s^{-1})	k'_x	K'_{app} (M)
$k_{cat.}$	$(1.1 \pm 0.2) \times 10^6$	$(9.1 \pm 2.2) \times 10^{-3}$ s^{-1}	109.90 ± 0.08
k_2	$(4.4 \pm 2.8) \times 10^2$	18.6 ± 1.7 M^{-1} s^{-1}	108.96 ± 0.29

$$k_{cat.}^{obs} = \frac{k_{cat.}{}^{s}_{s}K_{MeOH}[H^+] + k'_{cat.}({}^{s}_{s}K'_{app})^2}{[H^+]^2 + ({}^{s}_{s}K'_{app})^2} \quad (6)$$

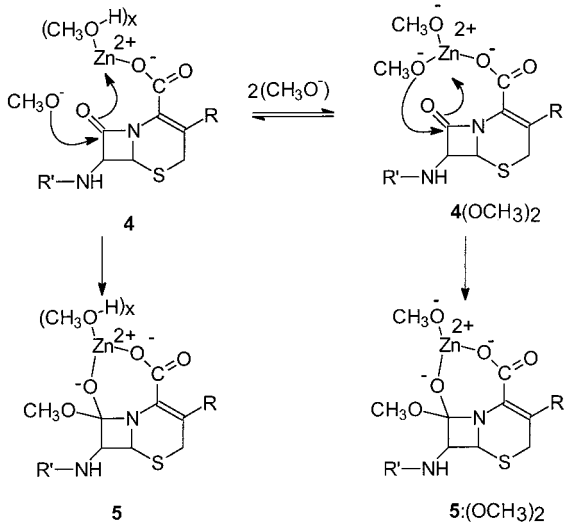
$$k_2^{obs} = \frac{k_2({}^{s}_{s}K_{app})^2[H^+]^2 + k'_2({}^{s}_{s}K_{app})^2({}^{s}_{s}K'_{app})^2}{([H^+]^2 + ({}^{s}_{s}K_{app})^2)([H^+]^2 + ({}^{s}_{s}K'_{app})^2)} \quad (7)$$

We deal first with the reactions of the Zn^{2+}:**1** complex(es) (these being represented in Scheme 1 as $k_{cat.}$ and $k'_{cat.}$), and subsequently with the reaction of a those same complex(es) with a second Zn^{2+} (represented in Scheme 1 as k_2 and k'_2).

(c) One-Zn^{2+} Mechanisms: Reactions of Zn^{2+}:1. The saturation behavior exhibited in the k_{obs} vs [Zn^{2+}] plots for methanolysis of **1** (e.g. Figure 3) and the linear dependence of the log $k_{cat.}^{obs}$ vs $^{s}_{s}$pH plot up to $\sim^{s}_{s}$pH 10 (Figure 4) suggests a process wherein external methoxide attacks a Zn^{2+}:**1** complex, a process similar to what is reported for the Zn^{2+} and Cu^{2+} promoted hydrolysis of benzyl penicillin[10] and to what we have observed before for the Zn^{2+}-promoted methanolysis of acetylimidazole.[15b] The maximal second-order rate constant for methoxide attack on the Zn^{2+}:**1** complex ($k_{cat.} = 1.1 \times 10^6$ M^{-1} s^{-1}) is roughly 9×10^5-fold larger than uncatalyzed methoxide attack on **1**, pointing to the strong Lewis acid role for the bound Zn^{2+} as shown in Scheme 2, **4** → **5**. By way of comparison, the Zn^{2+}-bound form of benzyl penicillin is activated toward HO^{-} attack by 4×10^5-fold (6.0×10^4 vs 0.15 M^{-1} s^{-1}, respectively).[10b,c] At $^{s}_{s}$pH values higher than 10, the log $k_{cat.}^{obs}$ vs $^{s}_{s}$pH plot turns downward which is explained by binding of two methoxides to the **1**:Zn^{2+} complex to form **1**:$Zn^{2+}(CH_3O^{-})_2$, thus decreasing the metal ion's Lewis acidity and concomitantly the attack of external methoxide. The general appearance of the plot gives a hint that at $^{s}_{s}$pH > 11 there is a developing plateau indicative of a spontaneous reaction of **1**:$Zn^{2+}(CH_3O^{-})_2$, $k'_{cat.} \sim 9 \times 10^{-3}$ s^{-1}, possibly attributable to internal delivery of CH_3O^{-} from the coordinated $Zn^{2+}(CH_3O^{-})_2$ as in **4**:$(OCH_3)_2$ → **5**:$(OCH_3)_2$, Scheme 2, or

(27) We have evidence that the carboxylate:Zn^{2+} association constant and K'_{app} values for titration of the carboxylate:$Zn^{2+}(CH_3OH)_x$ vary with the acidity of the carboxylic acid. Thus for 2×10^{-3} M 2,4-dinitrobenzoic acid, which may be a closer analogy to the nitrocefin molecule than acetic acid because of its greater acidity, the $^{s}_{s}pK_a$ is 6.55 ± 0.14 in the absence of metal ion. In the presence of 2×10^{-3} M $Zn(ClO_4)_2$, the $^{s}_{s}pK_a$ of the acid drops to 5.59 ± 0.08 and the two $^{s}_{s}pK_a$ values for 2,4-DNB:$Zn^{2+}(CH_3OH)_x$ are 9.90 ± 0.22 and 10.62 ± 0.25, giving a midpoint of the titration of $^{s}_{s}pK'_{app} = 10.26$. The computed binding constant, K_{assoc}, is 1×10^4 M^{-1}.

Scheme 2



its kinetic equivalent.[28] That process, while not very well defined from the data since these do not extend above $^{s}_{s}$pH 11.34 for technical reasons, is roughly 100-fold more efficient that the general base delivery of $HOCH_3$ promoted by the carboxylate of uncomplexed **1**, $k_{MeOH} = 8.96 \times 10^{-5}$ s^{-1}.

(d) Two-Zn^{2+} Mechanisms: Reactions of Zn^{2+}:1 with $Zn^{2+}(^{-}OCH_3)_2$. The involvement of a second Zn^{2+} in the methanolysis of Zn^{2+}:**1** is readily apparent from the k_{obs} vs $[Zn^{2+}]$ plots at all $^{s}_{s}$pH values as a linear section following the saturation attributable to the formation of Zn^{2+}:**1**. As stated above, the gradient of that line gives the k_2^{obs} constants at each $^{s}_{s}$pH, while the intercept gives the $k_{cat.}^{obs}$ values which can be taken as the rate constant for methanolysis of Zn^{2+}:**1** in the absence of the second Zn^{2+}. That the $k_{cat.}^{obs}$ values determined in this way do not relate to any process without an affiliated Zn^{2+} is evident from the fact that the intercepts throughout the $^{s}_{s}$pH region from 7.95 to 11.34 (the plateau region for uncatalyzed methanolysis of **1**), are all substantially larger than the spontaneous process (by 10^{2-}-10^{4}-fold, depending on the $^{s}_{s}$pH, Table 1). Curiously the K_{assoc} data in Table 1, referring to the kinetically determined association constants for the Zn^{2+}:**1** complex, increase with $^{s}_{s}$pH suggesting that CH_3O^{-} association with the complex actually increases the binding of **1**, possibly through a decrease in the coordination number of the Zn^{2+} when acquiring additional (−)-charged ligands. Above $^{s}_{s}$pH 10, the kinetic data do not allow exact determination of K_{assoc} because the plots of k_{obs} vs $[Zn^{2+}]$ are strictly linear and show no indication of curvature even at the lowest $[Zn^{2+}]$ employed (5×10^{-5} M), indicating that at these $^{s}_{s}$pH values the lower limit for K_{assoc} is $\sim 40 \times 10^{3}$ M^{-1}.

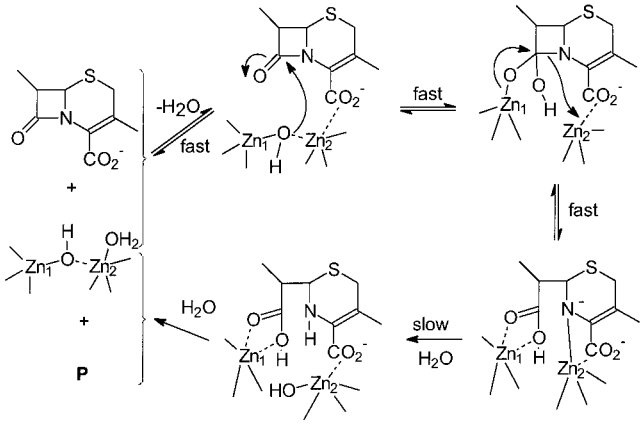
The ascending portion of the log k_2^{obs} vs $^{s}_{s}$pH profile is second order in $[CH_3O^{-}]$ and so requires that the second reactive Zn^{2+} must have two methoxides associated with it, undoubtedly as $Zn^{2+}(^{-}OCH_3)_2$. Furthermore, since the plateau region in the log k_2^{obs} vs $^{s}_{s}$pH profile commences at $\sim^{s}_{s}$pH 10, all kinetically important processes above that point must either become independent of $[CH_3O^{-}]$, or involve two or more species, the relative concentrations of which depend on a delicate counterbalancing of increasing and decreasing $[CH_3O^{-}]$. The most likely process consistent with all the above involves $Zn^{2+}(CH_3O^{-})_2$ reacting with both Zn^{2+}:**1** and Zn^{2+}:**1**:$(CH_3O^{-})_2$ as in Scheme 1. We have determined the k_2^{obs} values at all $^{s}_{s}$pH values under conditions of sufficiently high $[Zn^{2+}]$ that all the nitrocefin is complexed as Zn^{2+}:**1** or Zn^{2+}:**1**:$(CH_3O^{-})_2$. Since we do not observe a second saturation behavior at $[Zn^{2+}]$ up to 3 mM, there is no evidence for equilibrium binding between the second Zn^{2+} and Zn^{2+}:**1** at these concentrations. Thus the k_2 and k_2' terms in Scheme 1 and Table 2 are simply presented as second-order rate constants, the values of which determined from the NLLSQ fits of the data to eq 7 are 440 and 19 M^{-1} s^{-1}, respectively.

Table 2 reveals some interesting comparative data for (1) the rate constants for attack of $Zn^{2+}(CH_3O^{-})_2$ and CH_3O^{-} on **1**:Zn^{2+} ($k_{cat.} = 1.1 \times 10^{6}$ M^{-1} s^{-1} vs $k_2 = 440$ M^{-1} s^{-1}), (2) the rate constants for attack of $Zn^{2+}(CH_3O^{-})_2$ on Zn^{2+}:**1** or Zn^{2+}:**1**:$(CH_3O^{-})_2$ ($k_2 = 440$ M^{-1} s^{-1} vs $k_2' = 19$ M^{-1} s^{-1}), and (3) the values of K'_{app} computed from eqs 6 and 7 ($pK'_{app} = 9.90 \pm 0.08$ and 8.96 ± 0.29, respectively). The former can be accounted for by considering that the direct attack of methoxide on **1**:Zn^{2+} benefits greatly from electrostatic attraction of oppositely charged reactants,[29] which is not the case for neutral $Zn^{2+}(CH_3O^{-})_2$ reacting with **1**:Zn^{2+}. The latter two computed values for the pK'_{app} pertaining to the formation of Zn^{2+}:**1**:$(CH_3O^{-})_2$ might be considered different and thus indicative of an incomplete mechanistic model, but we have no additional experimental evidence that can be brought to bear on this possibility. We must note that we have used as a constant for the NLLSQ fitting, the titrimetric K_{app} determined in the presence of 0.01 M $N(n\text{-}Bu)_4ClO_4$, which may be different from the K_{app} extant in the kinetic runs where the ionic strength and $^{s}_{s}$pH are controlled with secondary and tertiary amine:$HClO_4$ buffers, perhaps contributing some errors to the computed constants. Nevertheless, given the inherent errors in the rate constants, the limited number of kinetic data, and the computed errors in the pK'_{app} values in Table 2, at the 95% confidence level we cannot say these values are different. This in turn suggests that the minimal mechanism we have presented in Scheme 1 is satisfactory to fit the available data. Based on that simplified mechanism, the computed rate constants for $Zn^{2+}(CH_3O^{-})_2$ reacting with **1**:Zn^{2+} and Zn^{2+}:**1**:$(CH_3O^{-})_2$ are quite similar. This suggests that reactivity differences that may be anticipated as arising

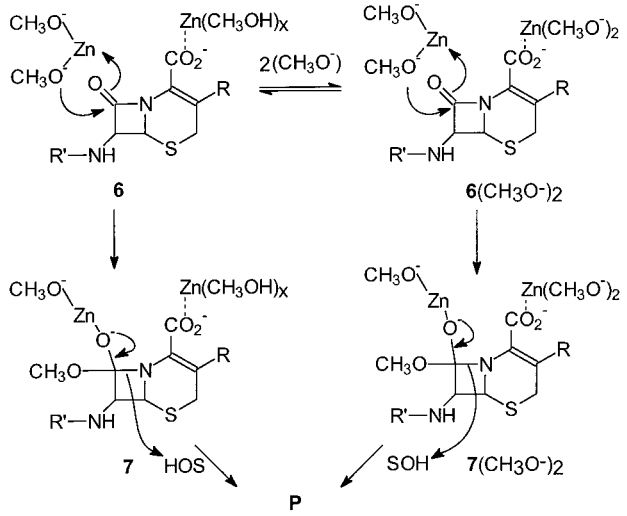
(28) A possible kinetic equivalent would be a general base delivery of methanol promoted by the coordinated $Zn^{2+}(CH_3O^{-})_2$. Alternatively, while not a kinetic equivalent, the process could represent attack of external CH_3O^{-} on **1**:$Zn^{2+}(CH_3O^{-})_2$, although this would require an eventual linear dependence on $[CH_3O^{-}]$ at higher $^{s}_{s}$pH. This remains speculative since we cannot provide evidence from the rate data because these do not extend far enough into the high $^{s}_{s}$pH region.

(29) In our previous study of the Zn^{2+} and La^{3+} promoted methanolysis of acetylimidazole, and its ligand exchange inert complex, $(NH_3)_5Co^{III}$:acetylimidazole, we noted strong electrostatic acceleration of methoxide on the metal-coordinated species and electrostatic deceleration of $M^{x+}(CH_3O^{-})_n$ reaction with the positively charged Co^{3+}-complex.[15b]

Scheme 3



Scheme 4



from differences in Lewis acidity in the **1**:Zn^{2+} and Zn^{2+}:**1**:$(CH_3O^-)_2$ complexes are minor, probably due to the intervention of a dual role mechanism for the attacking $Zn^{2+}(CH_3O^-)_2$ involving nucleophilic delivery of methoxide and stabilization of the developing (−) on the C═O, much the same as is proposed to be the case in the Zn^{2+}−β-lactamases.[7b]

Conclusions and Relevance to the Zn^{2+}−β-Lactamases

Benkovic and co-workers[7b] have noted that the Zn^{2+}−β-lactamases comprise a diverse collection of enzymes that are perhaps evolving under selective pressures. A low activity enzyme from *B. cereus* originally was identified as containing a single essential Zn^{2+} in the active site, and its rate-limiting step for β-lactam hydrolysis is reported to precede or incorporate C−N cleavage.[6] (Note however the recent crystal structure of a new crystalline form of *B. cereus* which contains two Zn^{2+} ions in the active site,[4e] but the second ion has a relatively low affinity and does not markedly enhance the activity). A more reactive enzyme from *B. fragilis* contains two essential Zn^{2+} ions and has $k_{cat.}/K_M$ values 10 times larger than for the *B. cereus* enzyme, at least for the substrate nitrocefin. In the latter case the slow step is not nucleophilic attack, nor cleavage of a tetrahedral intermediate, but protonation of the transiently formed anionic C−N cleavage intermediate.[7a] The proposed mechanism[7a] for lactam O═C−N cleavage, (summarized in Scheme 3) involves Zn_2 acting as a Lewis acid (possibly after binding with the substrate's COO^-) to enhance both nucleophilic attack and the leaving ability of the anionic N. Zn_1 serves to deliver a coordinated hydroxide, possibly stabilizing a transient anionic tetrahedral intermediate.

The lack of observed transient intermediates in the present work and the ready accessibility of the intermediates to solvent and buffering agents renders it unlikely that the slow step of the catalyzed methanolysis is any proton transfer. Rather, all the data support nucleophilic attack on the C═O or breakdown of a reversibly formed tetrahedral intermediate as rate limiting. Possible mechanisms for the two-Zn^{2+} processes are shown in Scheme 4, where $Zn^{2+}(CH_3O^-)_2$ serves to deliver a metal-bound methoxide to Zn^{2+}:**1** or Zn^{2+}:**1**:$(CH_3O^-)_2$. From our data we cannot provide a critical role for the carboxylate-bound Zn^{2+} in the two-Zn mechanism shown in Scheme 4 other than to (1) neutralize the charge of the substrate COO^-, rendering the C═O more susceptible to nucleophilic attack, (2) act as a Lewis acid enforced by its proximity to the scissile C═O, and (3) possibly to coordinate the lactam N rendering it a better leaving group. The major catalysis is provided by the external $Zn^{2+}(CH_3O^-)_2$ acting as a bimolecular nucleophile on **6** or **6**$(CH_3O^-)_2$. Of course in the enzyme the juxtaposition of both Zn ions in the active site makes cooperative interaction between them much more efficient than in our simple model, but the general features in the latter bear strong resemblance to what is proposed for the enzyme.

To our knowledge, the above model study is the first to demonstrate two roles for Zn^{2+} in promoting the acyl transfer from a β-lactam to HOS and thus provides the first small molecule analogue mimicking some of the chemistry believed to occur in the enzyme's active site. Further reports from these laboratories will present studies where dinuclear complexes promote the methanolysis of **1**−**3** and related penicillin derivatives.

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Supporting Information Available: Tables of kinetic data for k_{obs} values for the spontaneous methanolysis of **1** and its methanolysis in the presence of varying [Zn^{2+}] as a function of $^{s}_{s}$pH. This material is available free of charge via the Internet, http://pubs.acs.org.

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