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Ternary Ligand–Zinc–Hydroxamate Complexes

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Because of the importance of the hydroxamic acid functional group in zinc protease inhibitors, we have measured the stability constants of the ternary complex LMG, where L is series of tridentate and tetradentate ligands containing amino, carboxylate, pyridyl, and/or imidazolyl groups as enzyme models and G is the guest molecule, acetohydroxamate or *N*-methylacetohydroxamate. All measurements were determined by pH titration which gave reproducible and reasonable results. A general correlation between binding of LMG and that of LM showed ligands that strongly chelated zinc gave less LMG formation. Surprisingly, no correlation was observed between ligand charge and LMG formation even though the guest, acetohydroxamate, is anionic. The pH value of the maximum formation of the ternary complex is also correlated to the acidity of zinc-bound water; more acidic zinc-bound water results in a maximum ternary complex formation at lower pH value.

Introduction

The matrix metalloproteases (MMPs) are a class of hydrolytic enzymes necessary for tissue remodeling and the healing cascade.[1,2] Misregulated MMP activity can contribute to many disease states and conditions. Wounds possessing too much MMP activity may become ulcerated rather than heal properly.[3] Psoriasis may result from MMP action on healthy skin,[4] multiple sclerosis may result from MMP-mediated degradation of myelin,[5] loss of collagen from cartilage may result in rheumatoid arthritis,[6] and loss of collagen from bone may result in osteoporosis.[7] MMPs are also important for angiogenesis,[8] which is essential for the vascularization and growth of tumors. As a result, MMPs are therapeutic targets for slowing or halting the progression of tumor growth.[9−11] The inhibition of MMPs has received great attention.[12]

Prominent among the successful MMP inhibitors is the utilization of the hydroxamate functional group as the zinc binding group (ZBG).[13] In solution, the hydroxamic acid group is an ambident acid with similar pK_a's for the terminal OH and the NH, both in the range 8−10. Labeling studies (^{15}N) have shown that hydroxamate binds to MMP-3 as the conjugate base of an O-acid.[13] The active site of a MMP is typically a zinc ion coordinated in a tetrahedral fashion with three His or two His and one Asp/Glu, with a water molecule occupying a fourth site. X-ray crystallographic data for metalloenzyme−inhibitor complexes have shown that the hydroxamate functional group binds in a bidentate fashion to the zinc ion via its hydroxyl and carbonyl oxygens,[14] and

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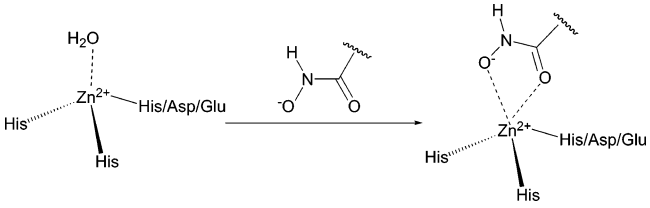

Figure 1. Active sites and inhibition of hydrolytic zinc enzymes.

expands the zinc coordination number from 4 to 5 (Figure 1). Thus, the hydroxamates typically form stable 5-membered chelates with zinc ion. For complexation of hydroxamate to Zn(II) in aqueous solution, the log $K = 5.4$;[15] in comparison, coordination of carboxylate occurs with log $K = 0.8$.

The hydroxamate unit is a structural feature of many drugs with promising applications toward the treatment of these diseases, yet side effects remain severe in some cases. Peptidic hydroxamate inhibitors have been developed that demonstrate excellent in vitro potency against MMPs. Most of the available compounds display broad-spectrum activity with equal potency against most members of the MMP family, although some selectivity has been achieved.[16] Most suffer from poor oral bioavailability.[16]

Although structure determination of the ternary complexes containing enzyme, metal, and inhibitor is extensive in the literature,[14] and some ternary mixed-ligand model complexes have been reported,[17] only few model studies concerning thermodynamic stability have been published.[18] Models are used to observe the chemistry surrounding the zinc metal ion such as the thermodynamic stabilities which would be difficult to obtain from endogenous enzymes. Other information can come from model studies. The influence of certain donor atoms on zinc behavior can be clarified. The size and shape of the potential cavity can be adjusted and its effects observed. Constructing different bonding geometries can significantly affect kinetic and thermodynamic aspects of binding and catalysis. Models studies can lead to (1) the understanding of substrate and inhibitor recognition,[19] (2) the development of new inhibitors,[17] (3) the design of artificial enzymes,[20] and (4) the determination of the mechanism of an enzyme.[21]

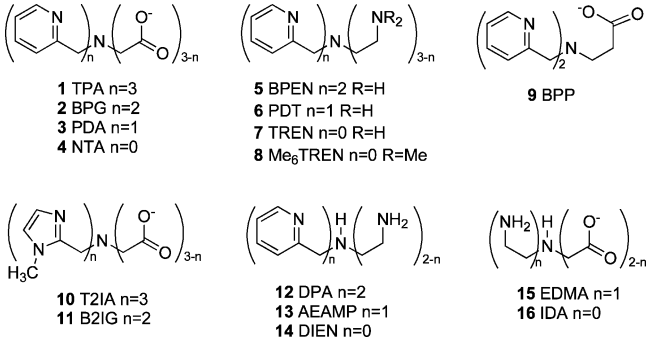

Figure 2. Ligands for studies.

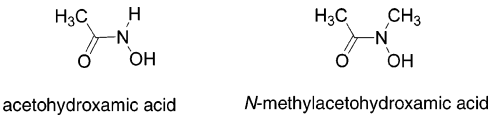

Figure 3. Hydroxamate inhibitors used in this study.

We have used a series of ligands containing amino, carboxylate, pyridyl, and/or imidazolyl groups as enzyme models.[21−23] Here we report ternary complexes composed of zinc chelating ligands, zinc(II), and hydroxamic acid derivatives as models to examine thermodynamic parameters that may be of relevance for inhibition of MMPs. The tetradentate and tridentate ligands **1**−**16** (Figure 2) together with acetohydroxamate and *N*-methylacetohydroxamate (Figure 3) were employed in this study. The binding of hydroxamates to zinc−ligand enzyme models was investigated by potentiometric titration in order to elucidate the effect of various coordination environments on inhibitor binding and develop strategies for discovering better inhibitors for drug design.

Experimental Section

Materials. All chemicals were of highest purity commercially available. Nitrilotriacetic acid (NTA), tris(2-aminoethyl)amine (TREN), diethylenetriamine (DIEN), iminodiacetic acid (IDA), and acetohydroxamic acid were obtained from Aldrich. Dipicolylamine (DPA) was purchased from TCI Americas, Inc. TPA,[24] Me_6TREN,[25] AEAMP,[26] and EDMA[27] were prepared according to literature procedures. BPG, PDA, BPEN, PDT, T2IA, B2IG were prepared as we earlier reported.[23] Purity was checked by ^{1}H NMR,^{13}C NMR, melting point, and/or elemental analysis.

***N*-Methylacetohydroxamic Acid.** *N*-Methylhydroxyamine−HCl salt (3.38 g), sodium bicarbonate (6.76 g), and diethyl ether (60 mL) were added to a 250 mL three-necked flask, equipped with a mechanical stirrer, and stirred at −78 °C. Acetyl chloride (3.18 g) in 5 mL of ether was added over a period of 10 min. The reaction mixture was maintained at −78 °C for 1 h, and then warmed to ambient temperature. The colorless ether layer was separated from the white precipitate. The minimum amount of water was added to dissolve the solid, and then the aqueous layer was extracted with ether and chloroform several times. The organic extracts were

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combined and dried over sodium sulfate. Removal of the solvent gave a yellow oil. The crude product was purified by column chromatography (silica, methylene chloride/diethyl ether 95:5, 2.75 g, 76% yield). The NMR spectrum in chloroform-*d* was consistent with the presence of both *E* and *Z* isomers.[28] ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.45 (s, 1H, br), 3.34 3.21 (s, 3H, *Z*/*E*), 2.10 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.6, 36.4, 20.4. Anal. Calcd for $C_3H_5NO_2$: C, 40.44, H, 7.92; N, 15.72. Found: C, 40.23; H, 8.18; N, 15.87.

3-[Bis(2-pyridylmethyl)amino]propionic acid (BPP), 9. To a 100-mL round-bottom flask was added β-alanine (1.52 g, 17 mmol), 2-picolyl chloride hydrochloric acid salt (5.59 g, 34 mmol), and 40 mL of water. To the stirred solution was added 3.41 g of NaOH (85 mmol) in 10 mL of water over a period of 10 min. The reaction mixture was stirred at 50−60 °C for 16 h. It was then cooled to room temperature, concentrated HCl was added to adjust pH to 6, and the solution was extracted with 30 mL of chloroform three times. The combined organic solution was dried over sodium sulfate to give crude product, which was recrystallized from ethanol and diethyl ether to afford a yellow solid (2.10 g. 45% yield). Mp 116−118 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.56 (d, J = 4.0 Hz, 2H), 7.70 (td, J = 7.8, 1.8 Hz, 2H), 7.44 (d, J = 7.8 Hz, 2H), 7.27−7.20 (m, 2H), 4.07 (s, 4H), 3.12 (t, J = 6 Hz, 2H), 2.70 (t, J = 6 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.2, 156.5, 149.1, 138.1, 124.6, 123.5, 59.2, 50.4, 32.4. ^{1}H NMR (200 MHz, CD_3CN) δ 8.53 (d, J = 5.0 Hz, 2H), 7.72 (td, J = 7.8, 1.8 Hz, 2H), 7.38 (d, J = 7.8 Hz, 2H), 7.29−7.22 (m, 2H), 3.92 (s, 4H), 2.96 (t, J = 6.4 Hz, 2H), 2.53 (t, J = 6.4 Hz, 2H). ^{13}C NMR (50 MHz, CD_3CN) δ 173.5, 157.8, 149.3, 137.2, 124.1, 123.0, 59.3, 50.2, 32.0. MS (MALDI-TOF): 272.1 (M + 1). Anal. Calcd for $C_{15}H_{17}N_3O_2$: C, 66.40, H, 6.32; N, 15.49. Found: C, 66.13; H, 6.10; N, 15.37.

Potentiometric pH Titrations. Potentiometric studies were conducted with a Titrino 702 autotitrator (Brinkmann Instruments). A Metrohm combined pH glass electrode (Ag/AgCl) with 3 M NaCl internal filling solution was used. All potentiometric titrations were carried out at 4 mM ligand, with I = 0.10 ($NaClO_4$) at 25 °C. The Zn(II) solution was standardized by primary standard EDTA in a NaOAc/HOAc buffer with 1-(2-pyridylazo)-2-naphthol as an indicator. The NaOH solution was standardized against potassium hydrogen phthalate with phenolphthalein as an indicator. All solutions were carefully protected from air by a stream of nitrogen gas. The k_w value was chosen as 13.78 for 25 °C, 0.1 M $NaClO_4$. A Gran's plot using the NaOH solution found the carbonate content below the acceptable limit of 2%.[29] Ligands were isolated or purchased in neutral or protonated forms. About 100 points were collected for each titration. The equilibrium constants were calculated using the program BEST.[29] All σ-fit values, defined in the program, were smaller than 0.015. Species distributions were calculated using the program SPE. For the determination of these constants, at least two independent titrations were always made.

Results and Discussion

Determination of equilibrium binding constants in Zn(II) coordination complexes presents a challenge due to the lack of convenient spectroscopic handles. We found that it was possible to obtain the data by potentiometric methods. A typical experiment consisted of three titrations to pH > 11 plus data analysis. First, a solution of free ligand plus 3 equiv of acid was titrated with the 0.1 M NaOH solution to obtain proton dissociation constants. These constants are not reported here but were essential for data analysis.[23] Then a solution of free ligand plus 3 equiv of acid in the presence of $Zn(ClO_4)_2$ was titrated to determine ligand−metal, LM, binding constants and acidity of zinc-bound water, $LM(OH_2)$. The last solution titrated was identical to the second except for the addition of acetohydroxamic acid which increased the acid equivalents to 4. Stability of the ternary species, LMG, was determined from this solution. Figure 4 is a representation of the three titrations necessary to calculate the binding of acetohydroxamate to a particular ligand−zinc complex. It also illustrates the addition of Zn(II) and Zn(II) with acetohydroxamate and its effects on the titration curve of the free ligand. Similar curves were obtained for the other ligands.

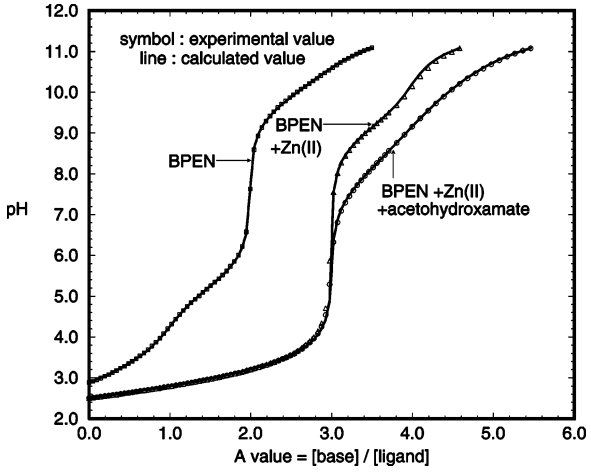

Figure 4. Typical titration curve ([ligand] = 5 mM).

The ligand protonation constants were determined from potentiometric titration of ligand·$3H^+$ (4 mM), using 0.1 M NaOH with I = 0.01 ($NaClO_4$) at 25 °C. The zinc−ligand complexation equilibria were determined from potentiometric titration of ligand·$3H^+$ (4 mM) in the presence of an equimolar or half amount of the zinc(II) ion under the same condition as the titrations of ligands. The ligand−zinc binding constants and zinc-bound water deprotonation constants were therefore obtained. Binary hydroxamate zinc systems were determined in the same way. The deprotonation constant and 1:1 zinc binding constant of acetohydroxamic acid are 9.22 and 5.40,[29] respectively, and those of *N*-methylacetohydroxamic acid are 8.70 and 5.10.[29] The ternary ligand−zinc−hydroxamate formation constants were obtained from titration of 1:1:1 ligand, zinc(II), and hydroxamic acid ion under the same conditions. The results are summarized in Table 1. The three logarithmic terms in Table 1 are defined by the following equations:

$$\mathrm{L + M + G \rightleftharpoons LMG} \qquad \beta_{LMG} = [LMG]/[L][M][G]$$

$$\mathrm{L + M \rightleftharpoons LM} \qquad \beta_{LM} = [LM]/[L][M]$$

$$\mathrm{LM + G \rightleftharpoons LMG} \qquad K = [LMG]/[LM][G]$$

$$\log K = \log \beta_{LMG} - \log \beta_{LM}$$

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Table 1. Thermodynamic Data for Ligand−Zinc Complexes and Hydroxamate−Zinc−Ligand Ternary Complexes

			G = acetohydroxamate			G = *N*-methylacetohydroxamate		
ligand	log β_{LM}[a]	pk_a of L−Zn−OH_2[a]	log β_{LMG}	log K	pH of max LMG	log β_{LMG}	log K	pH of max LMG
1 TPA[a]	11.00 ± 0.08[b]	8.03 ± 0.03[b]	14.63 ± 0.03	3.76	8.7	14.15 ± 0.03	3.27	8.4
2 BPG	11.4 ± 0.1	9.11 ± 0.03	15.2 ± 0.1	3.75	9.2	14.78 ± 0.04	3.38	8.9
3 PDA	10.89 ± 0.07	9.62 ± 0.08	14.492 ± 0.001	3.60	9.4	14.80 ± 0.08	3.92	8.9
4 NTA	10.53 ± 0.04	10.06 ± 0.07	13.90 ± 0.05	3.37	9.6	14.16 ± 0.03	3.63	9.2
5 BPEN	12.48 ± 0.02	9.145 ± 0.008	16.15 ± 0.03	3.66	9.2	15.74 ± 0.04	3.26	9.0
6 PDT	13.429 ± 0.007	9.63 ± 0.02	16.23 ± 0.02	2.80	9.4	16.00 ± 0.02	2.57	9.3
7 TREN	14.42 ± 0.05	10.21 ± 0.05	16.40 ± 0.01	1.98	9.7			
8 Me_6TREN	9.32 ± 0.02	8.61 ± 0.02	12.0 ± 0.1	2.71	9.0			
9 BPP	10.33 ± 0.02	10.05 ± 0.03	14.27 ± 0.01	3.94	9.4	13.98 ± 0.09	3.65	9.2
10 T2IA	11.98 ± 0.01	8.72 ± 0.05	15.16 ± 0.02	3.18	8.8			
11 B2IG	11.18 ± 0.01	8.99 ± 0.01	14.22 ± 0.04	3.04	9.0			
12 DPA	8.12 ± 0.05	8.59 ± 0.06	13.47 ± 0.09	5.34	8.6			
13 AEAMP	8.64 ± 0.01	8.9 ± 0.1	13.42 ± 0.05	5.08	9.0			
14DIEN	8.57 ± 0.04	8.87 ± 0.07	13.58 ± 0.09	5.01	9.0	13.45 ± 0.04	4.88	8.7
15 EDMA	8.11 ± 0.01		12.13 ± 0.05	4.02	8.5	12.28 ± 0.05	4.17	8.5
16 IDA	7.07 ± 0.02	8.8 ± 0.02	11.09 ± 0.02	4.02	8.9			

[a] Reference 23. [b] Reference 30.

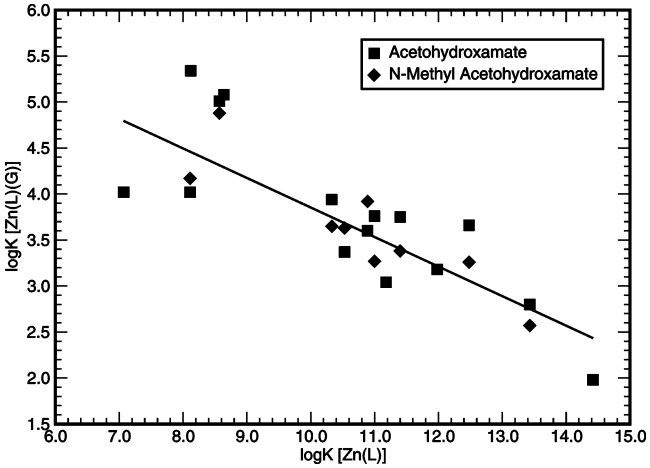

Figure 5. Relationship between binding constants for ternary Zn(L)(G) vs binary Zn(L) complexes. Me_6TREN was not included due to its unique steric properties.

The pH of maximum LMG is the pH value where the most LMG complex is formed for a particular ligand−zinc(II)−hydroxamate system, according to species distribution curves (vide infra).

We have shown in prior work that ligands with strong zinc binding show reduced zinc bound water acidity.[23] Figure 5, hydroxamate binding versus ligand−zinc stability, also shows a linear relationship, regardless of the ligand charges. Strong ligand−zinc binding gave weak ternary complex formation, while weaker ligand−zinc binding gave stronger ternary complex formation. Tetradentate ligands TPA, BPG, PDA, and NTA display similar zinc binding constants, and similar hydroxamate binding, although they bear different charges. Very strong LM binding (as with TREN) gave little overall ternary complex, LMG, formation. Tridentate ligands show smaller log β_{LM} and show larger ternary complex formation log K than tetradentate ligands. However, the data points for complexes with tridentate ligands show the greatest deviation from the line in Figure 5. A greater variety of solution structures is possible for tridentate ligand−Zn complexes with hydroxide or hydroxamate as compared with analogous complexes involving tetradentate ligands. In light of the overall trends, we propose that the main determination of acetohydroxamate binding is the stability of the LM species and not its charge.

All of ligands discussed here give five-membered chelation to zinc(II) ions except BPP, which forms a six-membered metallochelate from its central nitrogen atom and the oxygen atom of the carboxylate pendant group. Since it is known that five-membered chelation is more effective than six-membered chelation,[31] the zinc-binding constant of ligand BPP (10.33) is smaller than BPG (11.4). Although the Zn(BPP) complex has a less acidic zinc-bound water (pK_a = 10.04) than that of Zn(BPG) (pK_a = 9.11), for the ligand−zinc complex with hydroxamate binding, BPP behaves similarly to other five-membered chelation ligands: that is, the stronger ligand−zinc binding, the less ligand−zinc hydroxamate binding. The ligand Me_6TREN gives a surprisingly low log K_{LMG} which is attributed to the steric hindrance of the six methyl groups preventing bidentate chelation of acetohydroxamate.[22]

Species distribution curves provide an effective visual tool. For instance, Figure 6 shows a large difference in the maximum percent formation of ternary complex between the tetradentate ligand TPA, and the tridentate ligand DPA. Generally, a large log K value implies a large fraction of LMG formation; the maximum fractions of LMG for TPA and DPA are 39% and 88%, respectively. This results from a competition between hydroxide (OH^-) and hydroxamate (G^-) for complexing the LM species. Another way of stating this is that LM species with especially acidic LM−OH_2 gives less ternary complex, LMG, formation. It is shown in the species distribution curve of TPA that a significant rise occurs in the LM(OH) species at around pH = 6, a relatively acidic region, compared to the tridentate system, and therefore limits LMG formation. We found similar results comparing TREN with DIEN. In the competition for binding hydroxamate versus hydroxide, hydroxide will eventually dominate as pH increases. More acidic LM−OH_2 complexes are also better

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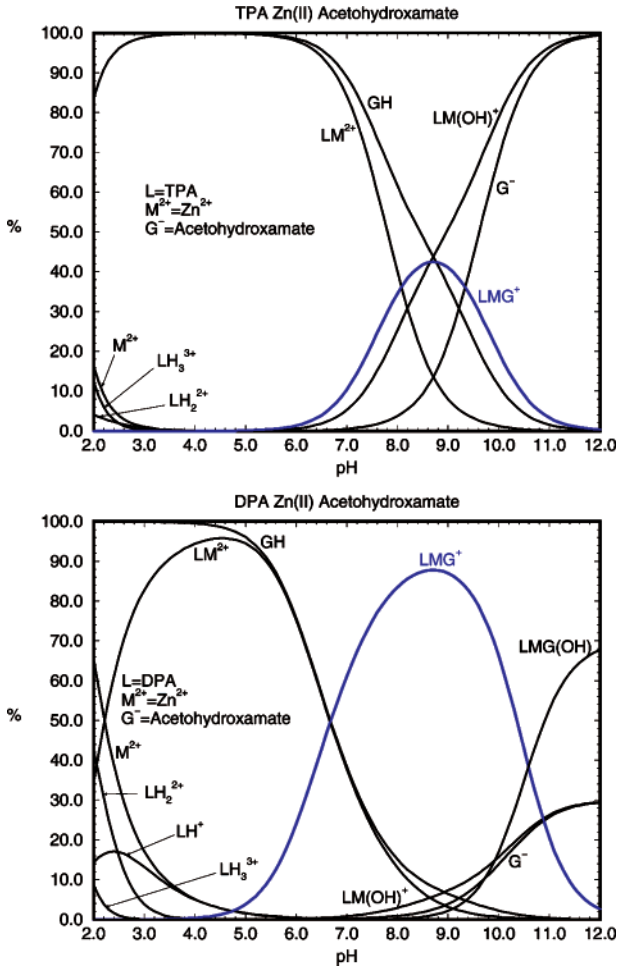

Figure 6. Species distribution curve TPA and DPA complexes with Zn(II) and hydroxamate.

OH binders, so that this competition will be more effective at lower pH.

Some debate in the literature persists as to whether hydroxamic acid is an O-acid or an N-acid.[32] X-ray crystallographic data have shown the hydroxamate functional group binds in a bidentate fashion to zinc with its two oxygen atoms,[14] but another novel binding mode of trifluoroacetohydroxamate to thermolysin has been found.[33] Although the 1:1 zinc complex of acetohydroxamate is 0.3 log unit more stable than that of *N*-methylacetohydroxamate, stability constants (log *K*) of two guest hydroxamate molecules toward ligand−zinc host systems are similar. This indicates the hydroxamate functional groups act as an O-acid in both cases,

(32) Monzyk, B.; Crumbliss, A. L. *J. Org. Chem.* **1980**, *45*, 4670−4675.
(33) Scolnick, L. R.; Clements, A. M.; Liao, J.; Crenshaw, L.; Hellberg, M.; May, J.; Dean, T. R.; Christianson, D. W. *J. Am. Chem. Soc.* **1997**, *119*, 850−851.

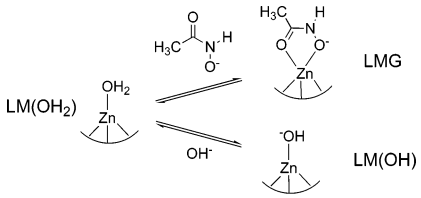

Figure 7. Formation of $LM(OH_2)$ (enzyme model), LM(OH) (activated enzyme model), and LMG (inhibited enzyme model).

since no deprotonation can occur from the nitrogen acid of *N*-methyl acetohydroxamic acid.

A correlation was found between the hydroxamate binding and the pK_a of zinc-bound water acidity. Ligands with more acidic zinc-bound water gave maximum LMG formation at lower pH due to competition by hydroxide; all of the ligands follow this trend. Increased zinc-bound water acidity means greater hydroxide affinity of the LM complex. Thus, the formation of LM(OH) can either be seen as the loss of an acidic hydrogen from $LM(OH_2)$ or the binding of hydroxide by LM (Figure 7). The hydroxamate must compete with hydroxide on LM binding, and the competition will be more severe for more acidic metal complexes. *N*-Methylacetohydroxamic acid is more acidic than acetohydroxamic acid; therefore, the maximum concentrations of ternary complexes form at a lower pH when the guest molecule is *N*-methylacetohydroxamate.

Conclusion

The potentiometric titration method produced reproducible and reasonable binding constants for the formation of ternary, mixed-ligand complexes of zinc. A correlation was observed for log *K* versus log β_{LM} where strong LM binding gave weaker LMG formation. The maximum formation of the ternary complex is limited by competition with hydroxide complexation (acidity of $LM(OH_2)$). The charge of the ligand confers relatively little influence on acetohydroxamate binding under the aqueous conditions studied. The pH value of the maximum formation of the ternary complex also correlates to the acidity of zinc-bound water; more acidic zinc-bound water results in maximum ternary complex formation at lower pH value. These considerations should be of interest in the rational design of zinc enzyme inhibitors.

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