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A Water-Soluble Bis(thiosemicarbazone) Ligand. A Sensitive Probe and Metal Buffer for Zinc

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The first member of a water-soluble family of bis(thiosemicarbazone) ligands is reported. It forms a 1:1 complex with Zn^{II} that absorbs intensely in the visible region (λ_{max} = 414 nm; ε = 1.8(4) $\times$ 10^4 M^{-1} cm^{-1}; pH 7.3). Its affinity for Zn^{II} (K_D = 5.9(3) $\times$ 10^{-9} M at pH 7.3) was determined by competition with ligand ethylene glycol *O,O′*-bis(2-aminoethyl)-*N,N,N′,N′*-tetraacetic acid. Its potential application as a chromophoric probe was demonstrated by estimation of the Zn^{II} binding affinities of the soluble metal-binding domains of two plant metal-transporting proteins.

Historically, quantitative detection of zinc at low concentrations is problematic because the d^{10} configuration of Zn^{II} normally precludes sensitive magnetic and spectroscopic properties. This has restricted in vivo study of zinc metabolism, but significant progress has now been achieved via the design of chromophoric ligand probes and, in particular, of fluorescent probes.[1−6] In comparison, sensitive and reliable zinc probes based on absorption in the visible region are less available.[7,8] Such probes have advantages in the quantification of binding stoichiometries and affinities in isolated biomolecules. The two probes, mag-fura-2 (Mf2) and 4-(2-pyridylazo)resorcinol (Par), are used widely in such applications but have limitations.[9] In addition, it is important to develop a range of probes with varying affinities to meet the requirements of different biomolecules.

Tetradentate bis(thiosemicarbazone) ligands form complexes with Cu^{II} and Zn^{II} that are excellent chromophores with high extinction coefficients in the visible region. A number of copper and zinc complexes of these ligands have attracted interest as promising metallodrugs and radiopharmaceuticals.[10−15] A significant advantage is that tetradentate ligands have a lower probability of forming ternary complexes with proteins, a potential complication for bidentate ligand probes.[16] This reduces uncertainties of interpretation, although equilibria can be slower to establish. A major hindrance in the application of bis(thiosemicarbazone) probes is their limited solubility in aqueous solutions.[17] The synthesis and characterization of the first member of a new class of water-soluble bis(thiosemicarbazone) ligands, $[H_2L]^-$ (Figure 1a), are reported here. The promise of this new ligand as a quantitative probe for Zn^{II} is demonstrated by its use in estimation of the affinities for Zn^{II} of the N-terminal metal-binding domains of two plant transporter proteins.

A four-step synthesis of the dimethylammonium salt $[Me_2H_2N][H_2L]$ is presented in Scheme S1 in the Supporting Information (SI). The reaction involves introduction of an aromatic sulfonate functional group to the bis(thiosemicarbazone) framework via a selective transamination reaction of diacetyl-4,4-dimethyl-4′-methylbis(thiosemicarbazone) with sulfanilic acid.[18] The product countercation $[Me_2H_2N]^+$ may be exchanged with other cations via ion-exchange chromatography. The new ligand is colorless and water-soluble. Reaction with aqueous zinc acetate immediately produced a bright-yellow-orange solution. The same reaction in *N,N′*-dimethylformamide, followed by precipitation with

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(1) Frederickson, C. J. *Int. Rev. Neurobiol.* **1989**, *31*, 145.
(2) Jiang, P.; Guo, Z. *Coord. Chem. Rev.* **2004**, *248*, 205.
(3) Que, E. L.; Domaille, D. W.; Chang, C. *J. Chem. Rev.* **2008**, *108*, 1517.
(4) McRae, R.; Bagchi, P.; Sumalekshmy, S.; Fahrni, C. *J. Chem. Rev.* **2009**, *109*, 4780.
(5) Thompson, R. B. *Curr. Opin. Chem. Biol.* **2005**, *9*, 526.
(6) Nolan, E. M.; Lippard, S. J. *Acc. Chem. Res.* **2009**, *42*, 193.
(7) Batista, R. M. F.; Oliveira, E.; Costa, S. P. G.; Lodeiro, C.; Raposo, M. M. M. *Org. Lett.* **2007**, *9*, 3201.
(8) He, S.; Iacono, S. T.; Budy, S. M.; Dennis, A. E.; Smith, D. W., Jr.; Smith, R. C. *J. Mater. Chem.* **2008**, *18*, 1970.
(9) Zimmermann, M.; Clarke, O.; Gulbis, J. M.; Keizer, D. W.; Jarvis, R. S.; Cobbett, C. S.; Hinds, M. G.; Xiao, Z.; Wedd, A. G. *Biochemistry* **2009**, *48*, 11640.
(10) Kraker, A.; Krezoski, S.; Schneider, J.; Minkel, D.; Petering, D. H. *J. Biol. Chem.* **1985**, *260*, 13710.
(11) Petering, D. H. *Bioinorg. Chem.* **1972**, *1*, 255.
(12) Petering, D. H. *Biochem. Pharmacol.* **1974**, *23*, 567.
(13) Green, M. A.; Klippenstein, D. L.; Tennison, J. R. *J. Nucl. Med.* **1988**, *29*, 1549.
(14) Fujibayashi, Y.; Taniuchi, H.; Yonekura, Y.; Ohtani, H.; Konishi, J.; Yokoyama, A. *J. Nucl. Med.* **1997**, *38*, 1155.
(15) Vavere, A. L.; Lewis, J. S. *Dalton Trans.* **2007**, 4893.
(16) Hendrickson, K. M.; Geue, J. P.; Wyness, O.; Lincoln, S. F.; Ward, A. D. *J. Am. Chem. Soc.* **2003**, *125*, 3889.
(17) Xiao, Z.; Donnelly, P. S.; Zimmermann, M.; Wedd, A. G. *Inorg. Chem.* **2008**, *47*, 4338.
(18) Paterson, B. M.; Karas, J. A.; Scanlon, D. B.; White, J. M.; Donnelly, P. S. *Inorg. Chem.* **2010**, *49*, 1884.

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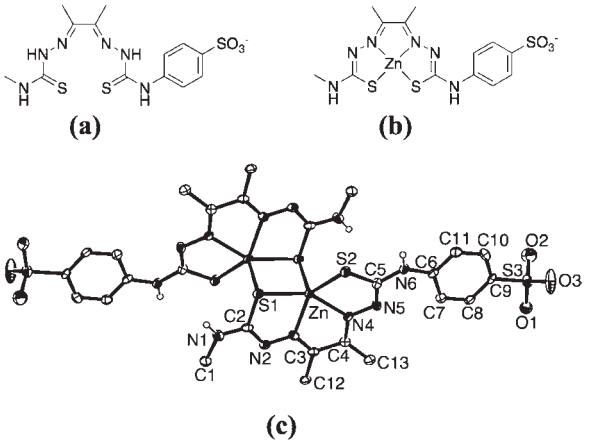

Figure 1. (a and b) Schematic presentations of $[H_2L]^-$ and $[Zn^{II}L]^-$ structures. (c) ORTEP representation (ellipsoids at the 40% level) of the X-ray structure of binuclear anion $[Zn^{II}L]_2^{2-}$ in crystals of $[Me_2H_2N]_2$-$[Zn^{II}L]_2 \cdot 2DMSO$. Bond length (Å): Zn−N3 2.102(7), Zn−N4 2.100(7), Zn−S1 2.443(3), Zn−S2 2.345(3), Zn′−S1 2.445(3). See Table S2 in the SI for other selected bond lengths and angles. The solvent was omitted for clarity.

diethyl ether, provided a yellow-orange solid as either the sodium or dimethylammonium salt. The anion was formulated as $[ZnL]^-$ featuring ligand L^{3-} (Figure 1b). Analysis of $[Me_2H_2N][ZnL]$ by single-crystal X-ray crystallography (Table S1 in the SI) revealed the presence of a dimer formed via sulfur atom S1 from an adjacent molecule acting as the donor in the apical position of an essentially square-pyramidal N_2S_3 coordination environment (Figure 1c). The internal ligand bond lengths indicate that, although there is extensive delocalization, the resonance form depicted in Figure 1b dominates with the C−S bonds, being more thiolate-like than thione-like [C5−S1 = 1.764(9) Å]. The formation of dimers in the solid state has been observed previously in zinc complexes of bis(thiosemicarbazonato) ligands, but it is likely that monomeric forms exist in solutions where coordinating solvents compete for the axial position.[19,20] In fact, electrospray ionization mass spectrometry (ESI-MS) analysis detected ion pairs assignable to both mononuclear and binuclear species but with mononuclear species dominating (Figure S1 in the SI).

Titration of $Na[H_2L]$ with Zn^{2+} in a 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer (20 mM; pH 7.3) induced an intense absorbance in the visible range (λ_{max} = 414 nm; $\varepsilon = 1.8(4) \times 10^4\ M^{-1}\ cm^{-1}$) with a tight isosbestic point at 361 nm (Figure 2). A plot of ε_{414} versus $[Zn^{2+}]/[H_2L^-]$ ratio produced a straight line with a sharp turning point at 1.0 (inset, Figure 2). A Job plot with varying ligand and zinc concentrations also confirmed a binding stoichiometry of 1:1 (Figure S2 in the SI).[21] These experiments demonstrated a clean conversion of the ligand into zinc complex(es) with identical absorption properties under the conditions. The X-ray crystal structure revealed a dimer anion, while ESI-MS detected both monomeric and dimeric forms, with the former being dominant. It is likely that both are present in an aqueous solution and that the position of the

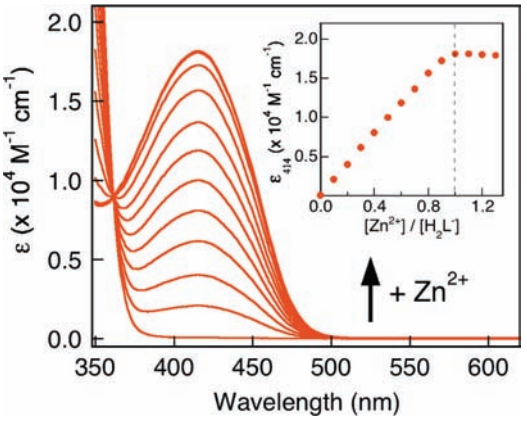

Figure 2. Change in the spectrum of a solution containing ligand $[LH_2]^-$ (60 μM) in a Mops buffer (20 mM; pH 7.3) upon titration with Zn^{2+} (1.0 mM). Inset: plot of ε_{414} vs $[Zn^{2+}]/[H_2L]^-$.

equilibrium depends on the solvent or the presence of other possible ligands such as buffers. However, a tight isosbestic point was observed in an aqueous Mops buffer (50 mM, pH 7.3; Figure 2), and the spectrum of the complex in this medium was stable and insensitive to the addition of anions Cl^-, HPO_4^{2-}, and SO_4^{2-}.[22] Ligand H_2L^- does not form colored complexes with Na^+, K^+, Mg^{2+}, or Ca^{2+}, and these ions do not interfere with the present application of in vitro determination of the affinities of pure isolated apoproteins for Zn^{II}. It does form colored complexes with Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}. Its application as a probe for these latter ions is being investigated.

In addition to its high molar absorptivity in the visible region, $[ZnL]^-$ fluoresces weakly in the visible, a characteristic that has been attributed to intraligand excitation (Figure S3 in the SI).[23] This intrinsic fluorescence has been used to track the intracellular distribution of certain bis(thiosemicarbazonato)zinc(II) complexes in human cancer cell lines.[20] The present new water-soluble zinc complex is fluorescent in an aqueous Mops buffer with an emission centered at λ_{em} = 540 nm following excitation at λ_{ex} = 420 nm. Importantly, the "free" ligand $[H_2L]^-$ is not fluorescent and the addition of Mg^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, or Cu^{2+} does not induce fluorescence but, as would be expected, the Cd^{II} complex emits similarly.

The binding affinity of the ligand for Zn^{2+} was estimated by competition with ligand $[H_2Egta]^{2-}$ [Egta = ethylene glycol *O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid] of known affinity at pH 7.3 ($K_D = 1.0 \times 10^{-9}$ M):[24]

$$[ZnL]^- + [H_2Egta]^{2-} \rightleftharpoons [H_2L]^- + [ZnEgta]^{2-} \quad (1)$$

$$\frac{K_{D(ZnL^-)}}{K_{D(ZnEgta^{2-})}} = \frac{([H_2L^-]_{total}/[ZnL^-]) - 1}{([H_2Egta^{2-}]_{total}/[ZnEgta^{2-}]) - 1} \quad (2)$$

Under conditions of effective competition, as reported by the A_{414} probe for $[ZnL]^-$, the *same* equilibrium position for eq 1

(19) Cowley, A. R.; Dilworth, J. R.; Donnelly, P. S.; Labisbal, E.; Sousa, A. *J. Am. Chem. Soc.* **2002**, *124*, 5270.
(20) Cowley, A. R.; Davis, J.; Dilworth, J. R.; Donnelly, P. S.; Dobson, R.; Nightingale, A.; Peach, J. M.; Shore, B.; Kerr, D.; Seymour, L. *Chem. Commun.* **2005**, 845.
(21) Job, P. *Ann. Chim. Appl.* **1928**, *9*, 113.
(22) The selection of buffers is quite important to metal ion binding. See: Yu, Q.; Kandegedara, A.; Xu, Y.; Rorabacher, D. B. *Anal. Biochem.* **1997**, *253*, 50.
(23) Xue, Z.-M.; Tian, Y.-P.; Wang, D.; Jiang, M. H. *Dalton. Trans.* **2003**, 1373.
(24) Martell, A. E., Smith, R. M., Eds. *Critical Stability Constants, Vol. 1: Amino Acids*; Plenum: New York, 1974; p 469.

could be approached from either side of the competition in ~40 min (Figure S4 in the SI). Although an equilibrium between mononuclear and binuclear forms may exist in solution, the experimental data from a series of experiments with varying Zn^{II} occupancies on both ligands were fitted to eq 2 satisfactorily (correlation coefficient $R > 0.99$), generating a consistent apparent binding affinity of ligand H_2L^- for Zn^{2+} at pH 7.3: $K_D = 5.9(3) \times 10^{-9}$ M (Table S3 and Figure S5 in the SI). This demonstrates that effective exchange of Zn^{II} between the two ligands occurs in solution according to eqs 1 and 2 and that any binuclear forms have little impact on the exchange and the position of equilibrium. This was further confirmed by the equivalent competition with two Zn^{II} binding protein domains (see below).

It is apparent that ligand $[H_2L]^-$ has potential as a chromophoric reagent for the determination of the affinity of proteins for Zn^{2+}. That potential was tested with the soluble N-terminal domains HMA4n and HMA7n (HMA = heavy-metal ATPase) from the membrane-bound P_{1B}-type ATPase HMA4 and HMA7 from the simple plant *Arabidopsis thaliana*. HMA4 is a zinc transporter essential for root-to-shoot translocation of zinc, while HMA7 is a copper transporter essential in ethylene signaling.[25,26] Both HMA4n and HMA7n bind 1 equiv of Zn^{II} with affinities around the nanomolar range, but their affinities differ by a factor of ~30.[9] Conditions of effective competition were surveyed for HMA4n and HMA7n (10−30 μM) at pH 7.3 (Figure 3).[28] The results demonstrate that, while ligand H_2L^- competes weakly with HMA4n for Zn^{2+} at comparable concentrations (30 μM), it competes effectively at a higher ligand concentration (180 μM) (Figure 3a and Table S4 in the SI). On the other hand, the affinity of HMA7n for Zn^{2+} is weaker and falls within the sensitive buffering range of H_2L^- for Zn^{2+}. Thus, ligand H_2L^- competes for Zn^{II} with HMA7n effectively at comparable concentrations (20−30 μM) and is a sensitive quantitative probe for the HMA7n protein (Figure 3b and Table S5 in the SI). Analysis of the experimental data in Tables S4 and S5 in the SI with an equation equivalent to eq 2 generated consistent affinity data for each protein domain at

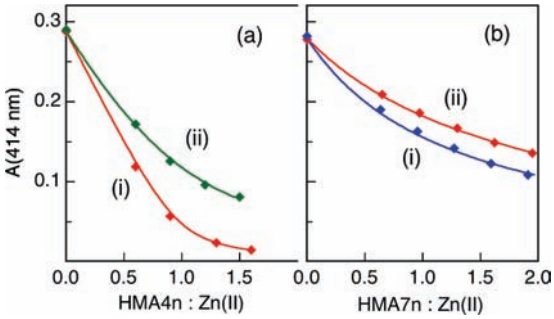

Figure 3. Determination of Zn^{II} dissociation constants (K_D) of HMA4n and HMA7n proteins in a Mops buffer (50 mM; pH 7.3; 100 mM NaCl) with probe $[H_2L]^-$. Decrease in A_{414} of solutions containing (a) $[Zn^{II}]_{total}$ = ~16 μM and $[H_2L^-]_{total}$ = 30 μM (i) or 180 μM (ii) with increasing relative concentration of HMA4n and (b) $[Zn^{II}]_{total}$ = ~15.5 μM and $[H_2L^-]_{total}$ = 20 μM (i) or 30 μM (ii) with increasing relative concentration of HMA7n. The experimental data were fitted directly to eq 2 satisfactorily ($R > 0.99$), generating the traces shown and the average K_D for each protein domain.

pH 7.3: K_D(Zn-HMA4n) = $1.6(1) \times 10^{-10}$ M and K_D(Zn-HMA7n) = $5.7(1) \times 10^{-9}$ M. These data are indistinguishable within experimental error from those values (2.1×10^{-10} M and 5.8×10^{-9} M) obtained previously.[9]

At pH 7.3, the affinity of ligand $[H_2L]^-$ for Zn^{II} ($K_D = 5.9 \times 10^{-9}$ M) is slightly higher than that of a popular Zn^{II} chromophore, Mf2 (2.0×10^{-8} M).[27] However, the Mf2 probe detects Zn^{II} binding via the absorbance of the free Mf2 ligand rather than that of the metal complex. Experimentally, this restricts variation of the ligand concentration and imposes an upper limit of ~35 μM on the total Mf2 concentration (cf. Figure S2 of ref 9). Consequently, for those proteins whose affinities for Zn^{II} are outside the sensitive Zn^{2+} buffering range of Mf2, it is difficult to establish an effective competition for Zn^{II} between this probe and the proteins, as was the case for HMA4n.[9] The present probe detects Zn^{II} binding via the absorbance of the metal complex rather than that of the free ligand. Consequently, variation of the ligand concentration over a much wider range is possible, extending the detection limit for the "free" Zn^{2+} concentration (cf. Figure 3a).

Manipulation of the functional groups of the ligand backbone will permit assembly of a family of ligands of varying affinity. This task is presently underway.

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Supporting Information Available: Crystallographic data in CIF format and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

(25) Sinclair, S. A.; Sherson, S. M.; Jarvis, R.; Camakaris, J.; Cobbett, C. S. *New Phytol.* **2007**, *174*, 39.
(26) Woeste, K. E.; Kieber, J. J. *Plant Cell* **2000**, *12*, 443.
(27) Simons, T. J. B. *J. Biochem. Biophys. Methods* **1993**, *27*, 25.
(28) The protein domains were isolated and reduced as reported in ref 9. To ensure the presence of effective competition equilibrium with fully reduced proteins, the reaction mixtures listed in Tables S4 and S5 in the SI were incubated under anaerobic conditions for at least 40 min in the presence of thiol reductant tris(2-carboxyethyl)phosphine (Tcep; 5 equiv) before *A*(414 nm) values were recorded. Controls without Tcep under the same conditions confirmed that Tcep has a negligible influence on the competition equilibria.