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Reaction of the zinc sensor FluoZin-3 with Zn_7-metallothionein: Inquiry into the existence of a proposed weak binding site

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ABSTRACT

It has been reported that Zn_7-metallothionein (MT), contains one weak binding site for Zn^{2+}. To test this conclusion, rabbit liver MT isolated at pH 7 was reacted with chelating agents of modest affinity for Zn^{2+}. Contrary to the previous study, no evidence was found for Zn^{2+} stoichiometrically bound to the protein with an apparent stability constant of about 10^8. Indeed, stability constant measurements based upon competition between Zn_7-MT and ligands of known stability with Zn^{2+} showed that all of the protein bound Zn^{2+} displayed the same stability constant at pH 7.4 and 25 °C of $(1.7 \pm 0.6) \times 10^{11}$. Brief reaction of Zn_7-MT with strong acid converted it into MT* and upon reneutralization into Zn_7-MT*, which demonstrated reactivity of about 1 Zn^{2+}/mol MT with competing ligands. Acid titration of Zn_7-MT to pH 2 or below rapidly resulted in the formation of Zn_7-MT* that displayed biphasic titration with base, revealing the rebinding of lower affinity Zn^{2+} between pH 5 and 7. Since MT is commonly acidified during preparation, care must be taken to document which form of the protein is present in subsequent experiments at pH 7.

1. Introduction

Metallothionein (MT) is a small, thiol rich metal binding protein that reacts with divalent metal ions such as Zn^{2+} and Cd^{2+} to form a two-domain conformation [1]. Each domain is comprised of a metal (M)-thiolate (S) cluster that organizes part of the polypeptide chain around it [1]. The N-terminal domain contains a M_3S_9 cluster, the C-terminal domain, a M_4S_{11} cluster. Each is characterized by a set of metal ions linked through bridging sulfhydryl ligands and to the peptide backbone through bridging and terminal thiolate ligands.

It is well established that MT contributes to protecting cells against the toxicity of Cd^{2+} and other metal ions through direct chelation [2]. Its multiplicity of sulfhydryl groups also inactivates a variety of oxidants and electrophiles [2]. Normally present in many cells as a zinc-protein and induced by Zn^{2+}, it has been hypothesized that metallothionein also is involved in intracellular Zn^{2+} trafficking [3–6]. One view is that MT simply acts as a transient Zn^{2+} storage site, buffering cells from changes in "free" or "loosely bound" Zn^{2+} [5].

$$7Zn^{2+} + \text{apo-MT} \rightleftharpoons Zn_7\text{-MT} \quad (1)$$

An extension has been to suggest that Zn_7-MT acts as a source of Zn^{2+} conversion of apo-proteins to Zn-proteins [7,8].

$$1/7Zn_7 - MT + \text{apo} - \text{protein} \rightleftharpoons Zn - \text{protein} + 1/7\,\text{apo} - MT \quad (2)$$

In this proposed reaction, the favorable stability constant of Zn-protein is sufficient to drive the equilibrium toward products.

Earlier reports of stability constant measurements of Zn_7-MT have only quoted values of greater than 10^{10} per Zn^{2+} at pH 7.4 [9,10]. That magnitude would allow for the observed reaction of Zn_7-MT with apo-carbonic anhydrase ($K = 10^{12}$) [7,11]. However, a stability constant of this size would preclude its reaction with apo-Zn-proteins of lower affinity for Zn^{2+}.

Recently, Maret and Krezel reported distinctly different results and concluded that apo-MT displays step-wise association of Zn^{2+} to form the holo-protein with the associated stability constants at spanning four orders of magnitude [12]:

$$Apo - MT + 4Zn^{2+} \rightleftharpoons Zn_4 - MT \quad K_{1-4} = 10^{11.8} \text{ per } Zn^{2+} \quad (3)$$
$$Zn_4 - MT + Zn^{2+} \rightleftharpoons Zn_5 - MT \quad K_5 = 10^{10.9} \quad (4)$$
$$Zn_5 - MT + Zn^{2+} \rightleftharpoons Zn_6 - MT \quad K_6 = 10^{10.7} \quad (5)$$
$$Zn_6 - MT + Zn^{2+} \rightleftharpoons Zn_7 - MT \quad K_7 = 10^{7.7} \quad (6)$$

Reaction (3) cooperatively constitutes the 4-metal cluster. Then, Zn^{2+} adds sequentially to the 3 metal cluster. With its range of stability constants, particularly K_7, Zn_7-MT could potentially donate Zn^{2+} to apo-proteins that weakly bind Zn^{2+}.

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The experimental determination of these stability constants began with the observation that the fluorescent Zn^{2+} sensor, FluoZin-3, reacts with Zn_7-MT to form a fluorescent Zn^{2+} product [12]. The FluoZin-3 structure includes a fluorophore linked to a set of amine and carboxylate metal binding ligands with an aggregate modest stability constant for Zn^{2+} at pH 7 of $10^{7.8}$ (Fig. 1) [13]. Data in support of the weak stability constants were obtained from two similar experiments. In the first, binding isotherms were fit to the titration of apo-MT with Zn^{2+} in the presence of FluoZin-3 and RhodZin-3. The second was based on competition between either FluoZin-3 or RhodZin-3 and MT for Zn^{2+}.

Besides their implications for metallothionein as a cellular participant in Zn^{2+} trafficking, the results presented by Maret and Krezel suggest that FluoZin-3 might be used to image the presence and intracellular location of Zn_7-MT. Considering the apparent centrality of MT-bound Zn^{2+} in cellular Zn^{2+} trafficking hypothesized by these authors and others, it was important to reexamine their determination that Zn_7-MT contains a weak Zn^{2+} binding site [3–6]. The results in the present paper address the validity of this conclusion and show that Zn_7-MT isolated at pH 7 from rabbit liver does not react significantly with FluoZin-3 and that Zn^{2+} ions but that the protein taken to low pH and then restored to pH 7 reacts as described by Maret and Krezel [12].

2. Experimental methods

2.1. Materials

Sephadex G-75, G-25, and G-15 were from GE Healthcare Bio-Sciences AB, DEAE-cellulose and 5,5′-dithio-bis(2-nitrobenzoic acid) were provided by Sigma. Nitrilotriacetate and 2-mercaptoethanol were obtained from Aldrich Chemical Co. FluoZin-3™ was obtained from Invitrogen. The source for Chelex-100 resin was BioRad. TCEP was from Hampton Research. The thiosemicarbazone ligands, 3-ethoxy-2-oxobutyraldehyde-bis(thiosemicarbazone) and 3-ethoxy-2-oxobutyraldehyde-bis(^{4}N-dimethylthiosemicarbazone) were gifts from Harold G. Petering.

2.2. Isolation of zinc metallothionein

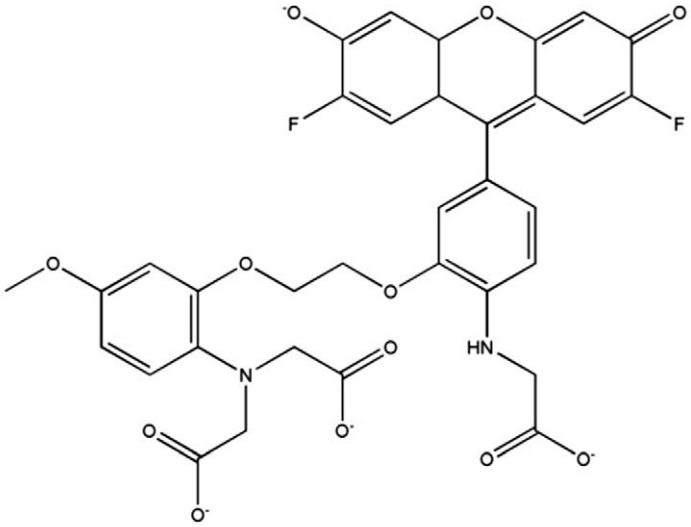
Female New Zealand white rabbits (5–6 lbs.) were injected with 2 mL of 0.15 M ZnSO4 every 24 h for eight consecutive days. On the eighth day, the rabbits were anaesthetized with ketamine followed by a lethal dose of sodium pentobarbital (120 mg/kg) into the heart. The livers were removed immediately from the rabbits and washed with cold saline to remove excess blood, divided into about 100 g samples and stored at −80 °C until further use. About 100 g of frozen rabbit liver was thawed and minced into small pieces. Tissue was mixed with homogenizing buffer (0.25 M sucrose, 0.01 M 2-mercaptoethanol, 5 mM Tris–Cl, and PMSF) and homogenized. The homogenate was centrifuged in a Sorvall high speed centrifuge (SS34 rotor at 20,000 rpm) for 20 min followed by ultracentrifugation in a Beckman L-70 ultracentrifuge (70TI rotor at 31,000 rpm) and the final supernatant was loaded onto a 4 × 85 cm Sephadex G-75 column equilibrated with 20 mM Tris–Cl, pH 7.4, and 5 mM 2-mercaptoethanol and eluted at 4 °C. The MT fractions were located by measuring the Zn^{2+} profile of the eluate. Altogether about 1–1.5 L of MT solutions were pooled. The two isoforms, MT1 and MT2, were separated by anion exchange HPLC using a semi-preparative DEAE column. The pooled MT solutions were loaded onto the column and MT was eluted with a gradient of 5–300 mM Tris–Cl at pH 7.4. MT-containing fractions were determined by measuring the concentrations of Zn^{2+} and thiol as described below. The overall yield of MT2 was 700–800 μg protein/g wet weight liver. Experiments described in the text used MT2 primarily and MT1 for some of the stability constant experiments.

2.3. Metallothionein characterization

Isolated MT was characterized by measuring the metal and sulfhydryl contents in the sample. The metal concentration was obtained by atomic absorption spectrophotometry and the sulfhydryl concentration was determined using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) assay [14]. The concentration of MT was also determined spectrophotometrically at 220 nm (ε_{220} = 1.59 × 105 M^{-1} cm^{-1}).

2.4. Preparation of modified metallothionein (MT*)

Concentrated HCl (12 N) was added to 1–2 mL of Zn_7-MT to a final concentration of 1.2 M HCl, reducing the pH to −0.4. The solution was left at low pH for 20 min and then neutralized to pH 7.5 by titration with 10 N KOH. In other experiments a range of hydro-



2,2'-(2-(2-(2-(carboxylatomethylamino)-5-(2,7-difluoro-3-oxido-6-oxo-6,9a-dihydro-4a*H*-xanthen-9-yl)phenoxy)ethoxy)-4-methoxyphenylazanediyl)diacetate

Fig. 1. Structure of FluoZin-3.