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A spectrophotometric method for the determination of zinc, copper, and cobalt ions in metalloproteins using Zincon

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ABSTRACT

Zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene) has long been known as an excellent colorimetric reagent for the detection of zinc and copper ions in aqueous solution. To extend the chelator's versatility to the quantification of metal ions in metalloproteins, the spectral properties of Zincon and its complexes with Zn^{2+}, Cu^{2+}, and Co^{2+} were investigated in the presence of guanidine hydrochloride and urea, two common denaturants used to labilize metal ions in proteins. These studies revealed the detection of metals to be generally more sensitive with urea. In addition, pH profiles recorded for these metals indicated the optimal pH for complex formation and stability to be 9.0. As a consequence, an optimized method that allows the facile determination of Zn^{2+}, Cu^{2+}, and Co^{2+} with detection limits in the high nanomolar range is presented. Furthermore, a simple two-step procedure for the quantification of both Zn^{2+} and Cu^{2+} within the same sample is described. Using the prototypical Cu^{2+}/Zn^{2+}–protein superoxide dismutase as an example, the effectiveness of this method of dual metal quantification in metalloproteins is demonstrated. Thus, the spectrophotometric determination of metal ions with Zincon can be exploited as a rapid and inexpensive means of assessing the metal contents of zinc-, copper-, cobalt-, and zinc/copper-containing proteins.

Introduction

Metals are indispensable constituents of approximately one-third of all proteins [1]. As such, metals are involved in virtually all biological processes, including metabolism, energy transduction, gene expression, cell signaling, formation of endo- and exoskeletons, and electron and information transfer [1,2].

Among the techniques suitable for the quantification of metal ions in metalloproteins, inductively coupled plasma mass spectrometry and atomic absorption and emission spectroscopies are likely to be the most widely employed [3]. However, although these techniques are reliable and sensitive, they suffer from the limitation of being rather costly (considering instrument acquisition and maintenance), time-consuming (with respect to sample preparation), and not always readily available [4,5]. Thus, simple spectrophotometric (or spectrofluorometric) techniques, which tend to be less costly and labor-intensive, are viable alternatives to those methods requiring more sophisticated instrumentation.

The determination of trace metals by ultraviolet–visible (UV–Vis)[1] spectroscopy typically relies on alterations in the absorption spectrum of a chromophoric chelator on binding the desired metal ion [6]. Among the many reagents eliciting complexation-induced spectral changes, the formazan dye Zincon (see Fig. 1) has been shown to serve as an excellent chromophore for the quantification of both zinc and copper ions in aqueous solution [6,7], two trace metal ions of particular relevance in biological systems. It is interesting to note that the possibility of using this chelator in the determination of metal contents in metalloproteins has remained largely unexplored. Indeed, there appears to be only one report pertaining to the use of Zincon as a spectrophotometric reagent for the determination of the metal content of a metalloprotein. In this case, the chelator was used in the assessment of the Zn^{2+} content of insulin preparations by a lengthy multistep procedure involving (i) acidification for sample clarification and protonation of Zn^{2+}-binding ligands, (ii) protein precipitation with trichloroacetic acid, (iii) centrifugation to separate the protein pellet, (iv) neutralization of the supernatant with NaOH, and (v) introduction of an aliquot of the sample into borate buffer containing Zincon [8]. It is important to note that the spectrophotometric determination of metal ions in metalloproteins using Zincon or other chromophoric chelators usually requires removal (or at least labilization) of these ions from the biological matrix prior to analysis. In the example outlined above, this process was achieved by protonation of the metal-binding ligands (see step i). Although such an approach may appear to be very simple, it requires

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[1] *Abbreviations used:* UV–Vis, ultraviolet–visible; GdnHCl, guanidine hydrochloride; SOD, superoxide dismutase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CyDTA, 1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid; PAR, 4-(2-pyridylazo)resorcinol.

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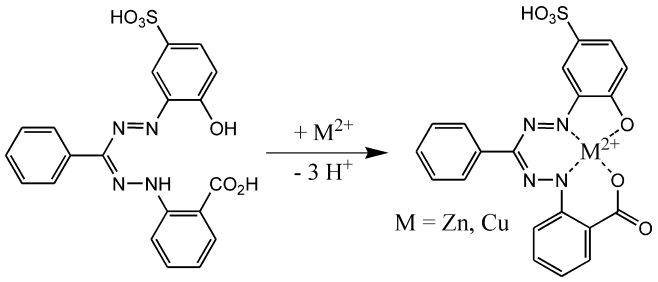

Fig. 1. Structures of Zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene) in its free and metal-bound forms.

acid neutralization and rebuffering at the final stages of sample preparation (see steps iv and v) because the detection of metal ions with most chromophoric chelators demands neutral or (slightly) alkaline conditions [6].

The use of chemical denaturants such as guanidine hydrochloride (GdnHCl) and urea constitutes an alternative approach to render the metal ions of a metalloprotein available for chelation [5,9,10]. The current article provides a comprehensive analysis of the influence of these denaturing agents on the absorption spectra of Zincon in its free and metal-bound forms. As a consequence of these investigations, an optimized method that allows the facile determination of Zn^{2+}, Cu^{2+}, and Co^{2+} is presented. Furthermore, a procedure for the quantification of both Zn^{2+} and Cu^{2+} in the same sample, which can be directly applied to assess the metal content of Zn^{2+}- and Cu^{2+}-containing proteins, is described. The prototypical Cu^{2+}/Zn^{2+}–metalloprotein superoxide dismutase (SOD) was chosen to demonstrate the effectiveness of this method of dual metal quantification.

Materials and methods

Chemicals

Boric acid, GdnHCl, 2-(*N*-morpholino)ethanesulfonic acid (Mes), sodium chloride, sodium perchlorate, tris(hydroxymethyl)aminomethane (Tris), and urea were purchased from BioShop (Burlington, ON, Canada). Ethanolamine, ethylenediaminetetraacetic acid (EDTA), and Zincon sodium salt (85% [w/w] dye content) were obtained from Sigma–Aldrich (St. Louis, MO, USA). SOD from bovine erythrocytes was purchased from MP Biomedicals (Solon, OH, USA). Transition metal salts were obtained as either sulfates (Zn^{2+}, Cu^{2+}) or chlorides (Co^{2+}, Ni^{2+}) from Fisher Scientific (Ottawa, ON, Canada). All buffers and solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA).

Stock solutions

A 1.6-mM stock solution of Zincon was prepared by dissolving 43.5 mg of the chelator (Na^+ salt, 85% dye content) in 1 ml of NaOH (1 M) prior to dilution to 50 ml with water. The stock solution was stable at 4 °C for (at least) 1 week.

Metal stock solutions (100 mM) of Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} were prepared by dissolving the appropriate amount of metal sulfates or chlorides in 100 ml of water. Working metal stock solutions were obtained from these 100-mM solutions by further dilution with water.

Stock solutions of boric acid (52.63 mM) containing urea (8.42 M), GdnHCl (4.21 or 6.32 M), NaCl (4.21 M), or $NaClO_4$ (4.21 M) were prepared by dissolving 325 mg of H_3BO_3 and the appropriate amount of denaturant (urea: 50.56 g; GdnHCl: 40.22 or 60.34 g) or salt (NaCl: 24.60 g; $NaClO_4$: 51.55 g) in water to achieve a final volume of approximately 90 ml. The pH of each solution was then adjusted to 9.0 using either NaOH (5 M) or HCl (6 M) prior to dilution of the mixture to 100 ml with water. The reference buffer containing only borate (52.63 mM, pH 9.0) was prepared analogously except for the omission of denaturant or salt.

A stock solution of SOD (140 μM) was prepared by dissolving the protein in water. The protein concentration was determined from its absorbance at 258 nm as described in the literature [11] as well as by the Bradford method [12].

Sample preparation and measurement

Samples (1 ml total volume) were prepared by adding 25 μl of a 40-fold stock solution of the desired metal to 950 μl of borate buffer (52.63 mM, pH 9.0). Reagent blanks were prepared analogously except for the substitution of the metal stock solution with water. For samples containing urea, GdnHCl, NaCl, or $NaClO_4$, a borate buffer containing the desired denaturant or salt was used. Complexation was initiated by the addition of 25 μl of the Zincon stock solution. Absorption spectra were recorded from 400 to 750 nm after 5 min of sample incubation at 20 °C on a BioChrom Ultrospec 2100 spectrophotometer (Cambridge, UK) using a cuvette with a 1-cm pathlength. The final concentrations of borate and Zincon in the samples were 50 mM and 40 μM, respectively. When present, the final concentrations were 8 M for urea, 4 or 6 M for GdnHCl, and 4 M for both NaCl and $NaClO_4$.

pH studies

The pH dependence of the absorption spectra of Zincon and its metal complexes in the absence and presence of denaturants was assessed in MTEN, a three-component buffer consisting of Mes (100 mM), Tris (50 mM), and ethanolamine (50 mM). The use of this buffer system allowed for the maintenance of ionic strength (I = 0.1 M) of the medium from pH 5.0 to 11.0 without the addition of salts [13].

A stock solution of MTEN (105.3 mM Mes, 52.63 mM Tris, and 52.63 mM ethanolamine) was prepared by dissolving 2.056 g of Mes, 638 mg of Tris, and 318 μl of ethanolamine in water to achieve a final volume of 100 ml. Aliquots of 5 ml of this stock solution were supplemented with either NaOH (5 M) or HCl (6 M) to adjust the pH to the desired value. In total, 13 buffer solutions ranging from pH 5.0 to 11.0 (in 0.5-pH intervals) were prepared. MTEN stock solutions containing urea (8.42 M) or GdnHCl (4.21 M) were prepared analogously except for the inclusion of 50.56 g of urea or 40.22 g of GdnHCl in the buffer.

Samples were prepared by adding 25 μl of a 1.6-mM metal ion stock solution to 950 μl of each of the 13 buffer solutions mentioned above. Complexation was initiated by supplementing the mixture with 25 μl of the Zincon stock solution to achieve a final concentration of 40 μM with respect to both the metal ion and the chelator. Following incubation of the samples for 5 min at 20 °C, absorption spectra were recorded as described above. Data from the 13 spectra were compiled into three-dimensional pH plots with the aid of PSI-Plot (Poly Software International, Pearl River, NY, USA).

Determination of Zn^{2+} and Cu^{2+} using EDTA

Both Zn^{2+} and Cu^{2+} were determined with Zincon using a two-step procedure involving the assessment of the total metal ion concentration followed by quantification of Cu^{2+} through demetallation of the Zn^{2+}–Zincon complex using EDTA. All samples (1 ml total volume) were prepared in borate buffer (50 mM, pH 9.0) containing urea (8 M) and Zincon (40 μM). Following incubation at 20 °C for 5 min, the absorbance at 615 nm (isosbestic point) was recorded. Two sets of standards, one containing Zn^{2+} (0–30 μM)