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Quenching of bathocuproine disulfonate fluorescence by Cu(I) as a basis for copper quantification

Viviana A. Rapisarda, Sabrina I. Volentini, Ricardo N. Farías, and Eddy M. Massa*

Departamento Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas, Consejo Nacional de Investigaciones Científicas y Tecnológicas, Universidad Nacional de Tucumán, and Instituto de Química Biológica "Dr. Bernabé Bloj", Chacabuco 461, San Miguel de Tucumán 4000, Argentina

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Abstract

In this paper we report the up to now ignored fluorescence properties of the specific Cu(I)–chelator bathocuproine disulfonate and their application in assays of total copper and Cu(I). The method is based on the linear quenching of the bathocuproine disulfonate emission at 770 nm (λ_{ex} 580 nm) by increasing concentrations of Cu(I), at pH 7.5. Copper concentrations as low as 0.1 μM can be determined. Other metal ions (iron, manganese, zinc, cadmium, cobalt, nickel) do not interfere. The procedure for total copper determination in proteins includes HCl treatment to release the copper, neutralization to pH 7.5 in the presence of citrate to stabilize the copper, and reduction of the copper to Cu(I) by ascorbate in the presence of the chelator. This assay gave results coincident with the analysis by atomic absorption spectroscopy in two selected proteins. In addition, conditions are described (omitting HCl treatment and reduction by ascorbate) for direct measurement of Cu(I) in native proteins, as illustrated for the *Escherichia coli* NADH dehydrogenase-2. Data show that the fluorometric assays described in this paper are simple and convenient procedures for total copper and direct Cu(I) quantification in determined biological samples.

Keywords: Copper assay; Cu(I) quantification; Bathocuproine disulfonate fluorescence; Spectrofluorometric method; NADH dehydrogenase-bound copper

Copper is a nutrient required for many biochemical and physiological functions. In humans this trace element is third in abundance, after Fe(III) and Zn(II), among the essential heavy metals [1]. In the body, copper shifts between the cuprous Cu(I) and the cupric Cu(II) forms and though the majority of the body's copper is in the Cu(II) form it is usually in the Cu(I) form inside of cells [2]. Recent studies [3] indicate that free copper ions are undetectable in cells and that all the copper is bound to proteins and low-molecular-weight substances. Copper acts as a cofactor for several enzymes such as lysil oxidase, cytochrome *c* oxidase, superoxide dismutase, dopamine β-hydroxilase, and tyrosinase [4]. Therefore, copper quantification is required in the course of many biological investigations.

In addition to classic atomic absorption spectroscopy, several techniques for copper quantification are available. Some of them require specific equipment, such as inductively coupled plasma atomic emission spectroscopy [5–7] and electrospray ionization mass spectroscopy [8]. An indirect fluorescence method for copper determination using thiobarbituric acid and 1,10-phenantroline was reported by Gutteridge [9]. Also the phenanthroline-based Phen Green FL indicator (Molecular Probe; P-6801) is a fluorescent reagent used as a general-purpose heavy metal sensor capable of detecting a broad range of metal ions, including both Cu(II) and Cu(I).

Bathocuproine disulfonate (BCS)[1] forms a specific 2:1 colored complex with Cu(I), whose molar absortivity at

* Corresponding author. Fax: +54-381-4248025.
E-mail address: massa@unt.edu.ar (E.M. Massa).

[1] *Abbreviations used:* BCS, bathocuproine disulfonate (2,9-dimethyl-4,7-diphenyl-l,10-phenanthrolinedisulfonic acid, disodium salt); SOD, superoxide dismutase; NDH-2, NADH dehydrogenase-2.

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480 nm is 13,500 [10–12]. This absorbance has been the basis for copper quantification in several protein studies, using the procedure described by Poillon and Dawson [12]. Another quantitative colorimetric assay for copper was reported by Brenner and Harris [13], employing bicinchoninic acid which forms a purple complex with Cu(I).

In this paper we report the up to now ignored fluorescence properties of BCS and describe a novel and sensitive method for routine copper determination in proteins, based on quenching of the BCS fluorescence by cuprous copper at neutral pH. The validity of the method was confirmed by determining the total copper content of HCl-treated erythrocyte SOD, obtaining the known copper/protein molar ratio of 2, as also determined by atomic absorption spectroscopy. In addition, the assay was suitable for direct quantification of thiolate-bound Cu(I) in the nondenatured fully active enzyme NADH dehydrogenase-2 (NDH-2) from *Escherichia coli*, as illustrated below.

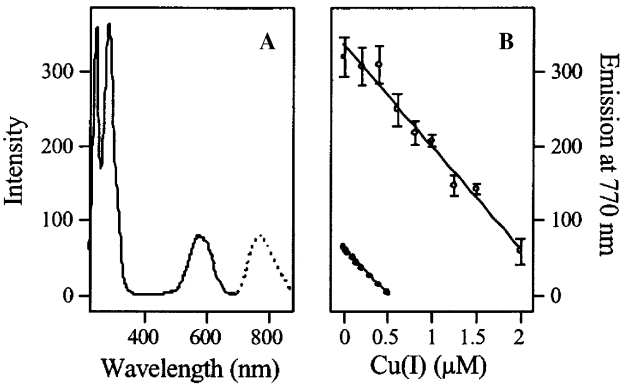

Fig. 1. Fluorescence properties of BCS. (A) Excitation (solid line) and emission (dotted line) spectra of 1 μM BCS in 50 mM phosphate buffer, pH 7.5, with $\lambda_{em} = 770$ nm and $\lambda_{ex} = 580$ nm, respectively. (B) Quenching of the BCS emission at 770 nm ($\lambda_{ex} = 580$ nm) by increasing concentrations of Cu(I) added as $CuCl_2$ in the presence of 10 μM ascorbate. BCS concentration: 1 μM (closed circles) or 4 μM (open circles).

Experimental procedures

Chemicals and enzymes. All chemicals used were analytical grade or in the purest commercially available form. Deionized/distilled water was used throughout. The copper reference solution (1 mg/ml) was from Boheringer–Mannheim (124 834). Stock solutions of 1 mM $CuCl_2$, $FeCl_3$, $FePO_4$, $ZnSO_4$, $MnSO_4$, $CoCO_3$, $NiCO_3$, and CdI_2 were prepared in 10 mM HCl. Bovine erythrocyte SOD (S2515) and BCS (B1125) were purchased from Sigma. Stock solutions of 20 mg/ml SOD and 5 mM BCS were stored frozen or at room temperature, respectively. NDH-2 was purified according to Jaworowsky et al. [14] by hydroxyapatite chromatography of solubilized membranes from *E. coli* IY85, as previously described [15]. The purified NDH-2 was stored at −70 °C in buffer A (1 M potassium phosphate, pH 7.5, containing 0.1% w/v of potassium cholate, 20 μM FAD, and 15% v/v of glycerol).

Fluorescence measurements. Fluorometric determinations were performed using either standard or submicro quartz cells (Starna Cells Inc.; 16.100-Q-10) for 100-μl samples. These cells were carefully washed and dried for each sample measurement. Excitation and emission spectra were recorded at 37 °C in a Shimadzu RF530 spectrofluorometer. Automatic baseline correction of the emission spectra was performed with the OMNIC software from Nicolet Instrument Corp.

Fluorometric assay of total copper in proteins. The method is based on the linear quenching of the BCS emission at 770 nm by increasing concentrations of Cu(I), shown in Fig. 1. The procedure for determining the total copper content in soluble proteins has three steps: release of the copper at pH < 1 [16], neutralization in the presence of citrate which stabilizes the copper, and reduction of the copper to Cu(I) by ascorbate in the presence of the chelator BCS. Standard copper samples are run in parallel with the test samples, in the same medium and conditions. The amount of copper in the test samples is calculated from their emission at 770 nm (λ_{ex} 580 nm) in comparison with the standard curve of BCS fluorescence versus copper concentration. This procedure was used to quantify the copper contents of erythrocyte SOD and *E. coli* NDH-2 as follows. Triplicate aliquots of the proteins (0.5 μM) and the standard $CuCl_2$ (0–2 μM) in 130 μl of medium (1 M phosphate buffer, pH 7.5) containing 20 mM sodium citrate (3 μl of 1 M stock solution) were acidified to pH < 1 by addition of 15 μl of concentrated HCl. The samples were then clarified by centrifugation and the supernatants were neutralized to pH 7.5 with 7.5 μl of 20 M NaOH. The resulting pH was checked by using pH strips. Just before the spectroscopic measurement, 4 μM BCS and 10 μM freshly prepared sodium ascorbate were added to the samples (the ascorbate concentration should not exceed 10 μM to minimize BCS fluorescence quenching due to the reductant). Under these conditions all the copper in the samples is reduced to Cu(I) and chelated by BCS, resulting in quenching of the chelator fluorescence in a magnitude that is proportional to the copper concentration. Thus, the amount of copper in the test samples was determined from their emission at 770 nm, with excitation wavelength of 580 nm, in comparison with the fluorescence of the standard copper samples.

Direct quantification of protein-bound Cu(I) by fluorometry. Under certain conditions, specific quenching of the BCS fluorescence can be applied to direct measurement of protein-bound Cu(I) (omitting the HCl treat-

ment and the reduction by ascorbate required for total copper determination), as illustrated here for the *E. coli* NDH-2. The adequate BCS concentration for a given protein amount was determined by recording the emission spectra of different BCS concentrations (from 0.2 to 4 μM) in the absence and the presence of 0.25 μM NDH-2. A plot of the emission decrease caused by the NDH-2 at each BCS concentration shows a plateau above 1 μM BCS (Fig. 2), indicating that a chelator concentration of 1 μM is suitable for the assay. Thus, the amount of Cu(I) in the protein was calculated from the emission decrease of 1 μM BCS in the presence of 0.25 μM NDH-2, in comparison with a plot of the 1 μM BCS emission versus standard Cu(I) concentrations, obtained under conditions identical to those for the test sample measurement.

The Cu(I) bound to the *E. coli* NDH-2 and to the chelator BCS is stable since precautions to avoid conversion to Cu(II) were not required. However, free Cu(I) in solution is easily oxidized to Cu(II) by oxygen unless anaerobic conditions are maintained.

Copper determination by atomic absorption spectroscopy. The total copper content of the SOD and NDH-2 samples was checked by atomic absorption spectroscopy. In the case of NDH-2, the samples were desalted by filtration through Sephadex G25 prior to the analysis. All the samples were acidified to pH < 1 by addition of concentrated HCl, to release the copper from the proteins. After clarification of the samples by centrifugation, the copper content of the supernatants was measured in a Z-5000 polarized Zeeman atomic absorption spectrophotometer by graphite furnace atomization analysis. Determinations were performed by triplicate in three preparations of SOD and NDH-2.

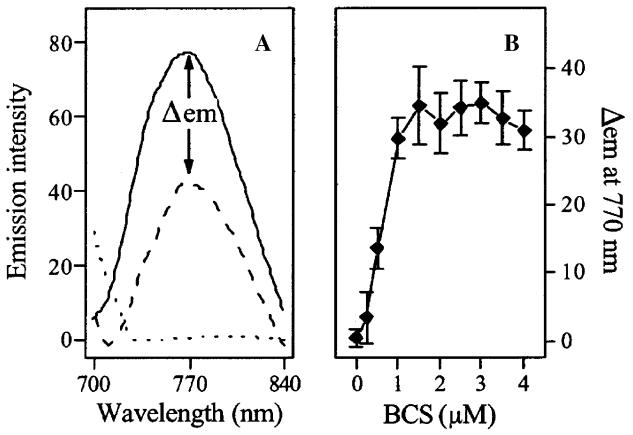

Fig. 2. Quenching of the BCS fluorescence by NDH-2. (A) Emission spectra ($\lambda_{ex} = 580$ nm) of 1 μM BCS in the absence (solid line) and the presence (dashed line) of 0.25 μM NDH-2. A control sample containing 0.25 μM NDH-2 in the absence of BCS is included (dotted line). (B) Plot of the decrease in the BCS emission at 770 nm ($\lambda_{ex} = 580$ nm) caused by 0.25 μM NDH-2 at the different BCS concentrations indicated on the abscissa. Measurements were performed in buffer A (the medium in which the purified NDH-2 was stored).

Protein determination. The protein content of the SOD and NDH-2 samples was measured using the Bio-Rad Protein Assay Kit II.

Results and discussion

Fluorescence properties of BCS and specific quenching by Cu(I). Although the absorbance at 480 nm of the 2:1 BCS/Cu(I) complex has been the basis for copper determination in several studies [12,17,18], the BCS fluorescence and its potential use in copper assays have not been recognized up to the present work. We discovered the BCS emission at 770 nm during our recent studies on the thiolate-bound Cu(I) of the *E. coli* NDH-2 [19] and developed the procedures described here to determine total copper and Cu(I) in the purified enzyme and in other proteins. These assays are simple, specific, and sensitive, as reported below. The technique is applicable to soluble samples containing copper accessible to the chelator, provided that the blank of medium alone is available to obtain the 100% value of the BCS emission and the plot of quenching by standard Cu(I) under the same conditions of the test. In general, the method is not applicable to crude sources unless nonspecific quenching of the BCS fluorescence by undetermined components of the samples can be discarded.

Fig. 1 shows the fluorescence properties of BCS at neutral pH. As can be seen (Fig. 1A), BCS exhibits an emission band with a maximum at 770 nm. The excitation spectrum, recorded at $\lambda_{em} = 770$ nm, presents three peaks: two in the UV region with maxima at 230 and 280 nm and the other in the visible region with a maximum at 580 nm (Fig. 1A). The emission studies reported in this paper were carried out with $\lambda_{ex} = 580$ nm to avoid the excitation of other fluorophores (e.g., tryptophyl residues and FAD) present in the proteins.

The BCS fluorescence at neutral pH is linearly quenched by increasing amounts of Cu(I), as shown in Fig. 1B, but not by Cu(II). Other metal ions such as Fe(III)/(II), Mn(II), Zn(II), Co(II), Cd(II), and Ni(II) do not interfere in the BCS fluorescence nor in the quenching by Cu(I), even when assayed at concentrations 10-fold higher than that of the copper (not shown). Specificity is an important parameter even in the case of purified proteins because they often bind more than one kind of metal ion. In acidic medium the BCS emission spectrum is different and quenching by Cu(I) is not observed. The specific quenching of the BCS emission by Cu(I) at pH 7.5 is the basis of the copper assays reported in this paper. As can be seen in Fig. 1B, the range of Cu(I) concentrations that can be determined depends on the chelator concentration: with 1 μM BCS the useful copper range is 0.1–0.5 μM (0.01–0.05 nmol of copper in 100-μl samples when using the submicro quartz cells), whereas with 4 μM BCS up to 2 μM copper (0.2 nmol in

100 μl) can be determined. For samples containing around 0.2 μM copper, greater sensitivity is obtained with 1 μM than with 4 μM BCS, since a greater percentage of quenching by the Cu(I) is observed with the lower chelator concentration.

Total copper determination in proteins. Data in Table 1 show that our assay based on quenching of the BCS fluorescence by Cu(I) and the analysis by atomic absorption spectroscopy gave coincident results in the quantification of the copper contents of erythrocyte SOD and *E. coli* NDH-2. The known copper/protein molar ratio of 2 [20] was obtained in the case of SOD. This enzyme is a dimer of identical subunits and contains two Cu(II) atoms per molecule [20]. For the NDH-2 a copper/protein molar ratio of 1 was determined (one copper atom per polypeptide chain of the enzyme).

Our initial attempts to determine the total copper content of the purified NDH-2 in buffer A (the medium in which the enzyme is stored) by atomic absorption spectroscopy were unsuccessful due to interference by the high salt concentration from the medium (1 M potassium phosphate). Desalting procedures had to be applied before HCl treatment of the NDH-2 samples to determine their copper contents by atomic absorption spectroscopy. Desalting led to loss of the enzymatic activity, which reflected a protein modification caused by this treatment and raised the possibility of a change in its copper content. Our novel fluorometric assay did not require desalting of the NDH-2 samples and, thus, it is an advantageous and rapid test of the copper content in particular biological systems (e.g., in chromatographic eluates where Na^+ or K^+ concentrations may be very high).

Direct Cu(I) quantification in the native NDH-2. Our previous studies [15] demonstrated that the *E. coli* NAD-2 catalyzes the reduction of Cu(II) to Cu(I) with NADH as electron donor. Recently, spectroscopic evidence indicating that purified NDH-2 contains thiolate-bound Cu(I) was obtained in our laboratory [19]. To quantify the Cu(I) bound to the active NDH-2 we developed the procedure described here. No other technique was available to fulfill this aim. FAD present in the NDH-2 samples interfered with methods based on the absorbance at 480 nm of the BCS/Cu(I) complex and the sensitivity of these methods was insufficient for the low concentration of the purified enzyme in our preparations.

As shown in Fig. 2A, the BCS emission at 770 nm is quenched by NDH-2, evidencing an interaction between the chelator and the Cu(I) from this enzyme. A protein that does not contain Cu(I), such as the erythrocyte SOD (whose copper remains in the cupric state in the absence of the substrate superoxide), has no effect on the BCS fluorescence under the conditions of the experiment in Fig. 2 even when the enzyme concentration is raised to 1 μM (not shown). The quenching effect of 0.25 μM NDH-2, measured as the difference in the BCS emission in the absence and the presence of the protein, increases as the BCS concentration is raised to 1 μM and remains constant from 1 to 4 μM BCS (Fig. 2B). Thus, a BCS concentration of 1 μM was used for quantifying the Cu(I) from 0.25 μM NDH-2, since this amount of chelator is sufficient for the copper present in the protein sample and gives maximal sensitivity in the emission quenching (highest percentage of decrease in the BCS emission in the presence of the protein). Data from three experiments such as that in Fig. 2 indicate that 0.25 μM NDH-2 causes a mean decrease of 29.7 ± 2.1 units in the BCS emission at 770 nm. This decrease is equal to that provoked by 0.25 μM standard Cu(I) under identical conditions (not shown). Thus, it appears that 0.25 μM NDH-2 contains 0.25 μM Cu(I), indicating a Cu(I)/protein molar ratio of 1 (there is one Cu(I) atom per each polypeptidic chain of NDH-2). Since the contents of Cu(I) and total copper in the purified NDH-2 are coincident, all the copper bound to the enzyme is in the cuprous state under the conditions of this study. It is relevant that the Cu(I) measurements were performed under conditions in which the NDH-2 enzymatic activity remains high.

This direct procedure can be used to determine the Cu(I) bound to other proteins, with the requisite that the Cu(I) be accessible to the chelator. Inaccessibility might result from the coordination characteristics and/or steric conditions of the Cu(I) in a particular protein.

Comparison of this technique with other methods. The sensitivity of our fluorometric procedure is about 50-fold higher than that of the colorimetric procedure based on the absorbance at 480 nm of the BCS/Cu(I) complex, which requires copper concentrations of at least 5 μM to obtain readings distinguishable from the blanks. Another disadvantage of the colorimetric method is the interference by substances absorbing around 480 nm, such as the enzyme cofactor FAD present in our NDH-2 samples.

The lowest limit of the detectable copper concentration range (around 0.1 μM) by our fluorometric assay is about sevenfold higher than that by graphite furnace atomic absorption spectroscopy. One major drawback to atomic absorption spectroscopy, and to other techniques that require more sophisticated instrumentation

Table 1
Determination of the total copper content of bovine erythrocyte SOD and *E. coli* NDH-2

Protein	Cu/protein molar ratio	
	Fluorometric assay	Atomic adsorption spectroscopy
SOD	1.91 ± 0.22	2.06 ± 0.23
NDH-2	1.09 ± 0.11	0.96 ± 0.08

Note. Values are means ± SD.

such as inductively coupled plasma atomic emission spectroscopy (which can determine multiple elements in the same sample), is that they do not identify the oxidation state of the element in its original matrix. This is also the case when our fluorometric technique is used for total copper determination. However, quenching of the BCS fluorescence by cuprous copper at neutral pH can also be advantageously used for direct quantification of Cu(I) in nondenatured proteins, as illustrated here, and could be adapted for measurements of Cu(I) in denatured proteins and other samples if changes in the copper valency during the assay process are prevented, e.g., as described in the procedure of Poillon and Dawson [12] or by maintaining anaerobic conditions to avoid oxidation by O_2.

As commented above, the fluorometric test may be advantageous compared to atomic absorption spectroscopy when samples containing high salt concentrations are assayed for copper that is usually present in very low amounts.

Conclusion

The fluorometric assays described in this paper are simple and convenient procedures for total copper and direct Cu(I) quantification in determined biological samples.

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