



Notes & Tips

A direct spectrophotometric method for the simultaneous determination of zinc and cobalt in metalloproteins using 4-(2-pyridylazo)resorcinol

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ARTICLE INFO

Article history:

Received 16 March 2009

Available online 10 May 2009

ABSTRACT

An assay involving the direct and simultaneous determination of low micromolar concentrations (1–10 μ M) of both zinc and cobalt ions suitable for metal content analyses of metalloproteins is described. The procedure exploits differences in the visible absorption spectra of the chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) resulting from its complexation to Zn^{2+} and/or Co^{2+} ions and is based on the fit of experimental spectra to a linear addition of Beer–Lambert law. The method eliminates the need for separating or masking one of the metal ions prior to their quantification and could prove to be particularly useful in studies on Co^{2+} -substituted zinc proteins.

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The spectrophotometric method involving the use of the chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR)¹ for the assessment of the metal ion content in metalloproteins has gained popularity during recent years because it is not only simple but also less costly and labor-intensive than those techniques requiring more sophisticated instrumentation [1–4]. However, the nonspecific nature of the interaction of PAR with many different divalent metal ions typically limits the chelator's use to cases where a metalloprotein contains only one type of metal. When two different ions are to be quantified with PAR, it is critical to either separate both species or mask one of the ions [5,6]. Here we describe a method for the direct and simultaneous quantification of both Zn^{2+} and Co^{2+} ions using PAR that exploits differences in the electronic spectra of the uncomplexed and metal-bound chelator.

Zinc is an indispensable constituent of many proteins involved in diverse cellular events such as metabolism, gene expression, and cell signaling [7,8]. Because Zn^{2+} is spectroscopically silent, insights into mechanistic aspects of zinc enzyme function and inhibition are often derived from studies on Co^{2+} -substituted forms of these proteins [9,10]. Indeed, Co^{2+} substitution has been successfully exploited to spectroscopically probe the active sites of a variety of mononuclear zinc proteins (e.g., carboxypeptidase A [11], thermolysin [12], angiotensin-converting enzyme [13]) and binuclear zinc proteins (e.g., aminopeptidases [14], metallo- β -lactamases [15]).

At the onset of any investigations on Co^{2+} -substituted zinc enzymes, the extent of metal substitution needs to be properly

assessed. This is particularly important when studying binuclear zinc enzymes using mixed ZnCo hybrids [14,15] or when Co^{2+} -substituted mononuclear zinc enzymes are generated either by direct exchange [10] or by expressing the protein in the presence of Co^{2+} in the cell culture. As outlined above, however, the quantification of two (or more) metal ions with PAR can be achieved only after separating or masking any interfering ions [5,6].

To simplify the quantification of Zn^{2+} and Co^{2+} , the possibility of exploiting spectral differences between PAR in its free and Zn^{2+} - and Co^{2+} -bound forms for the direct and simultaneous assessment of both metal ions was explored. Because the method reported here is aimed at determining the metal content of proteins, all samples were prepared in Hepes buffer (50 mM, pH 7.4) containing guanidine hydrochloride (GdnHCl) at a concentration (4 M) sufficient to unfold most proteins and, therefore, to render the metal ions accessible to chelation.

In mixtures containing PAR in its uncomplexed and complexed (with Zn^{2+} and/or Co^{2+}) forms, the total absorbance (A) at wavelength λ is given by

$$A_{\lambda} = d \times [\text{PAR}]_0 (x\epsilon_{\lambda}^{\text{PAR}} + y\epsilon_{\lambda}^{\text{PAR:Zn}} + z\epsilon_{\lambda}^{\text{PAR:Co}}) \quad \text{with } x + y + z = 1, \quad (1)$$

where d is the pathlength (in cm), $[\text{PAR}]_0$ is the total concentration of PAR; $\epsilon_{\lambda}^{\text{PAR}}$, $\epsilon_{\lambda}^{\text{PAR:Zn}}$, and $\epsilon_{\lambda}^{\text{PAR:Co}}$ are the molar extinction coefficients for PAR in its free and metal-bound states; x is the molar fraction of free PAR (i.e., $x = [\text{PAR}]/[\text{PAR}]_0$); y is the molar fraction of PAR complexing Zn^{2+} ($y = [\text{PAR:Zn}]/[\text{PAR}]_0$); and z is that bound to Co^{2+} ($z = [\text{PAR:Co}]/[\text{PAR}]_0$). Thus, the concentrations of Zn^{2+} and Co^{2+} in a given sample should be extractable from a fit of the corresponding spectrum to Eq. (1), with x , y , and z serving as fitting parameters.

Prior to fitting experimental spectra to Eq. (1), $\epsilon_{\lambda}^{\text{PAR}}$, $\epsilon_{\lambda}^{\text{PAR:Zn}}$, and $\epsilon_{\lambda}^{\text{PAR:Co}}$ were determined from the individual spectra of free PAR, PAR:Zn, and PAR:Co. As shown in Fig. 1A, the spectrum of

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¹ Abbreviations used: PAR, 4-(2-pyridylazo)resorcinol; GdnHCl, guanidine hydrochloride; PAR:Zn, PAR complexed to Zn^{2+} ; PAR:Co, PAR complexed to Co^{2+} .

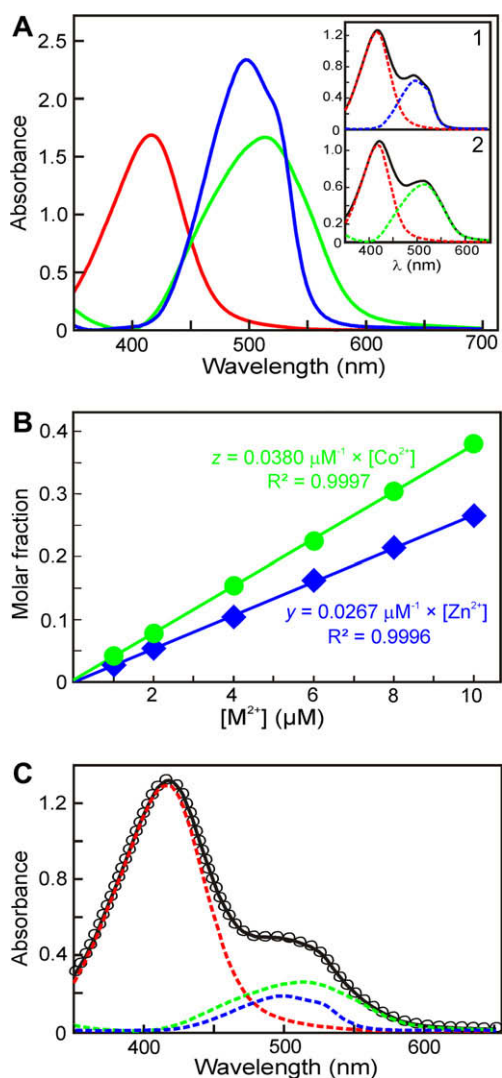


Fig. 1. Quantification of Zn^{2+} and Co^{2+} using PAR. All absorption spectra were recorded at 20 °C in Hepes (50 mM, pH 7.4) containing GdnHCl (4 M) and are represented as the mean of three independent experiments. Spectra were recorded on a Biochrom Ultraspec 2100 spectrophotometer (Cambridge, UK) using a cuvette of 1 cm pathlength. The initial concentration of PAR was 50 μM in each case. (A) Spectra of 50 μM free PAR (red), PAR:Zn (blue), and PAR:Co (green) complexes. The spectrum of PAR:Zn (blue dashed line) was estimated from that of PAR (50 μM) exposed to 10 μM Zn^{2+} (black line) by subtracting the spectral contribution of free PAR in such a way as to minimize the absorbance on the short-wavelength limb of the main absorption peak (inset 1). Using this approach, the experimental spectrum (black line) is best represented as the sum of spectra of 36.75 μM free PAR (red dashed line) and 13.25 μM PAR in complex with Zn^{2+} (blue dashed line). Inset 2 depicts the spectrum of PAR exposed to 10 μM Co^{2+} (black line), which can be represented as the sum of spectra of 31 μM free PAR (red dashed line) and 19 μM PAR in complex with Co^{2+} (green dashed line). (B) Dependence of the molar fractions y (for PAR:Zn) and z (for PAR:Co) on the metal ion concentration. PAR was exposed to Zn^{2+} (blue) and Co^{2+} (green) at the indicated concentrations for 5 min prior to recording absorption spectra. The y and z parameters were obtained by fitting the spectra to Eq. (1) using the spectral data shown in panel A. Trend lines were calculated by linear regression and are based on the mean of three independent experiments. Error bars were omitted because they are within the size of the symbols. (C) Absorption spectrum (circles) and spectral fit (solid black line) of a mixture of PAR (50 μM), Zn^{2+} (3 μM), and Co^{2+} (4 μM). Based on the obtained y and z parameters and the trend lines in panel B, the calculated concentrations of Zn^{2+} and Co^{2+} were 3.0 (± 0.1) and 3.9 (± 0.1) μM , respectively. The contributions of the individual spectra of free PAR (38.6 μM , red), PAR:Zn (4.0 μM , blue), and PAR:Co (7.4 μM , green) to the fit are depicted as dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

uncomplexed PAR (50 μM) was found to display an absorption maximum at 416 nm ($\epsilon = 33,700 \text{ M}^{-1} \text{ cm}^{-1}$), an observation in

accordance with the literature [1]. With respect to the spectrum of PAR:Zn, a preliminary titration of the chelator with Zn^{2+} (up to 1 mM) revealed that a clean spectrum of the complex could not be obtained due to the presence of significant amounts of free PAR in the mixture (see Supplemental Fig. 1 in supplementary material). Based on Eq. (1), however, spectra of PAR/ Zn^{2+} mixtures can be represented as the linear addition of its constituent spectra (i.e., those of free PAR and PAR:Zn). Thus, the spectrum of PAR:Zn was estimated from the spectrum of PAR (50 μM) exposed to Zn^{2+} (10 μM) by subtracting the spectral contribution of 36.75 μM free PAR (Fig. 1A, inset 1). The spectrum of PAR:Zn displayed an absorption maximum at 497 nm with an extinction coefficient, ϵ_{max} , of 46,700 $\text{M}^{-1} \text{ cm}^{-1}$. Because the complexation of 10 μM Zn^{2+} was found to require 13.25 μM PAR, the chelator appears to form a mixture of PAR: Zn^{2+} complexes with the stoichiometry of 1:1 (67.5%) and 2:1 (32.5%). For the sake of consistency, the spectrum of PAR:Co was estimated in a manner similar to that described for PAR:Zn by subtracting the spectral contribution of 31 μM free PAR from the spectrum of PAR (50 μM) exposed to 10 μM Co^{2+} (Fig. 1A, inset 2). The spectrum of PAR:Co showed an absorption maximum at 513 nm with an extinction coefficient, ϵ_{max} , of 33,300 $\text{M}^{-1} \text{ cm}^{-1}$. Because the complexation of 10 μM Co^{2+} required 19 μM PAR, the chelator appears to almost exclusively form a 2:1 complex with the metal ion. The spectra of PAR:Zn and PAR:Co, adjusted to reflect a concentration of 50 μM with respect to the chelator, are shown in Fig. 1A.

Following the determination of $\epsilon_{\lambda}^{\text{PAR}}$, $\epsilon_{\lambda}^{\text{PAR:Zn}}$, and $\epsilon_{\lambda}^{\text{PAR:Co}}$, absorption spectra of samples containing PAR (50 μM) and either Zn^{2+} or Co^{2+} (in the concentration range of 0–10 μM) were recorded from 350 to 650 nm and subsequently fit to Eq. (1) by the least-squares method using the Solver add-in within MS Excel with x , y , and z as fitting parameters and $x + y + z = 1$ as a constraint. As shown in Fig. 1B, the molar fractions y (for PAR:Zn) and z (for PAR:Co) were found to increase linearly with increasing metal ion concentrations.

To assess the accuracy and reliability of the method described here, spectra of a variety of samples containing known concentrations of Zn^{2+} and Co^{2+} were recorded and fit to Eq. (1) (see Table 1). Fig. 1C shows representative experimental and fitted spectra of PAR (50 μM) exposed to Zn^{2+} and Co^{2+} at concentrations of 3 and 4 μM , respectively. Following the spectral fitting, the Zn^{2+} and Co^{2+} concentrations were determined with the aid of the fitted molar fractions y and z and the trend line equations depicted in

Table 1

Accuracy of the simultaneous spectral determination method for Zn^{2+} and Co^{2+} .

Known concentration (μM)		Concentration extracted from fit (μM) ^a		Quality of fit ^b
Zn^{2+}	Co^{2+}	Zn^{2+}	Co^{2+}	$\sum \Delta^2$
1.0	2.0	0.9 (± 0.1)	2.0 (± 0.1)	0.0078
2.0	3.0	1.8 (± 0.2)	3.1 (± 0.1)	0.0192
2.0	6.0	1.9 (± 0.1)	6.1 (± 0.3)	0.0028
2.0	8.0	1.7 (± 0.1)	8.4 (± 0.4)	0.0495
3.0	4.0	3.0 (± 0.1)	3.9 (± 0.1)	0.0040
4.0	3.0	4.0 (± 0.1)	2.9 (± 0.1)	0.0050
5.0	5.0	4.9 (± 0.1)	5.0 (± 0.1)	0.0126
6.0	2.0	6.2 (± 0.2)	1.9 (± 0.2)	0.0253
7.0	2.0	7.1 (± 0.7)	1.9 (± 0.7)	0.0778
8.0	4.0	7.9 (± 0.1)	3.9 (± 0.6)	0.0045

Note. All samples were prepared in Hepes (50 mM, pH 7.4) containing GdnHCl (4 M), and spectra were recorded at 20 °C (350–650 nm, 1-nm intervals). A more comprehensive table is provided as Supplemental Table 1 in the supplementary material.

^a Each value represents the mean (\pm standard error of the mean) of three independent experiments.

^b The quality of fit (to Eq. (1)) is expressed as the mean of the sum of squared deviations.

Fig. 1B. In the case of the sample described above (Fig. 1C), the concentrations of Zn^{2+} and Co^{2+} extracted from the fit were $3.0 (\pm 0.1)$ and $3.9 (\pm 0.1)$ μM , respectively and, thus, were in excellent agreement with those expected. It is important to note that the quality of the fit was found to decrease significantly when the data were fitted using only the spectrum of PAR:Zn or PAR:Co (see Supplemental Fig. 2 in supplementary material). Thus, the fitting routine is capable of correctly identifying and quantifying the metal ions in the sample. As shown in Table 1, the calculated metal ion concentrations for all samples investigated were generally found to be in accordance with the known concentrations (typically within $\pm 10\%$ for Zn^{2+} and $\pm 5\%$ for Co^{2+}). Furthermore, the values for the standard error (typically $\leq 10\%$) appear to be a reflection of the method's fairly high degree of precision.

In conclusion, the method described here allows a reliable and direct assessment of the Zn^{2+} and Co^{2+} content of aqueous samples without the need for separating or masking one of these metal ions prior to analysis. Because the spectra of PAR in its free and complexed forms have previously been shown to be unaffected by the presence of proteinaceous material in the sample (Refs. [1,2] and own observations), the method can be directly applied to determine the Zn^{2+} and Co^{2+} content of metalloproteins harboring both metal ions. Furthermore, the general methodology described here may also be applicable to the simultaneous determination of both Zn^{2+} and Cu^{2+} or Ni^{2+} given that the spectra of PAR in complex with the latter metal ions deviate from that of PAR:Zn (see Supplemental Fig. 3 in supplementary material). Finally, an MS Excel spreadsheet containing the data shown in Fig. 1A and B, as well as the complete fitting routine, is available from the authors on request.

Acknowledgments

We thank the Natural Sciences and Engineering Research Council (NSERC) for financial support and Laurentian University for providing assistance through the Laurentian University Research Fund (LURF).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.05.007.

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