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Interaction of cupric ion with parvalbumin

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Abstract

Cod parvalbumin, a calcium-binding protein, possesses a specific Zn^{2+} (or Cu^{2+}) binding site per molecule. This work employed fluorescence energy transfer techniques to measure the distance between the Zn^{2+} (Cu^{2+}) site and the stronger Ca^{2+}-binding site in parvalbumin. Specifically, the distance between Tb^{3+} bound at the Ca^{2+} site and Co^{2+} bound to the Zn^{2+} (Cu^{2+}) binding site was 10.3 ± 0.9 Å. Lastly, the effects of Cu^{2+} on the physico-chemical properties of parvalbumin were studied by measuring the accessibility of protein thiol groups to 5,5′-dithio bis(2-nitrobenzoic acid) and by its affinity for the fluorescent probe 4,4′-bis[1-(phenylamino)-8-naphthalene sulfonic acid] dipotassium salt. The thiol group accessibility decreased and the affinity to the fluorescent probe increased upon complexation of Cu^{2+} to the protein. It appears that the binding of Cu^{2+} converts parvalbumin to an apo-like state.

Keywords: Parvalbumin; Fluorescence spectroscopy; Cupric ion binding; Calcium ion binding

1. Introduction

Parvalbumin (PA) [1] is a classical calcium-binding protein, the physico-chemical properties of which are well known [1]. This 12,000 molecular weight protein has an acidic isoelectric point (p*I* 4–5) and contains six α-helices, two pairs of which form two strong Ca^{2+} binding sites (CD and EF sites) [2]. These sites also bind Mg^{2+}, Na^+, K^+, and lanthanide ions. The physiological function of PA is still not thoroughly understood. Its concentration in the sarcoplasm of some muscle cells is rather high and correlates well with the speed of the muscle relaxation. Haiech and coworkers [3] suggested that PA serves as a soluble relaxing factor in fast muscle movement. Nevertheless, measurements of the Ca^{2+} and Mg^{2+} dissociation and association rate constants [4,5] suggest that this might probably not be the case, at least not during a single twitch, due to the slow rates of Ca^{2+} and Mg^{2+} dissociation.

Recently it was found that PA possesses an additional cation binding site which binds Zn^{2+},

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[1] Abbreviations used: bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalene sulfonic acid] dipotassium salt; DTNB, 5,5′-dithio bis(2-nitrobenzoic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethane sulfonic acid; PA, parvalbumin

Cu^{2+} or Co^{2+} ions [6,7]. The Cu^{2+}-affinity constant for PA is ca. 10^5 M^{-1} in the presence of Ca^{2+}, and ca. 10^6 M^{-1} in the absence of Ca^{2+}, which suggests some type of cooperative interaction between the two types of binding sites. The binding of Ca^{2+} to PA greatly increases its stability to temperature, pH and denaturants, while the binding of Cu^{2+} or Zn^{2+} ions to the Ca^{2+}-loaded protein decreases its stability to denaturation. The present work expands on these observations in a more detailed study which delineates the topographical relationship between the Cu^{2+} and Ca^{2+} sites, as well as effects on the physico-chemical properties of the protein.

2. Materials and methods

2.1 Protein

Parvalbumin was isolated from cod skeletal muscles according to previously published procedures [8]. The purity of the preparation was checked electrophoretically. Protein concentration was determined spectrophotometrically using an absorption coefficient $\epsilon_{280\,nm} = 7200$ M^{-1} cm^{-1} [1].

2.2 Chemicals

The following chemicals were purchased from commercially available sources: MES, Sigma Chemical Co.; HEPES, Boehringer Mannheim Biochemicals; cobalt chloride, Aldrich Chemical Co.; terbium chloride and DTNB, Sigma Chemical Co. DTNB concentration was estimated from its optical absorbance on a UVIKON 860 spectrophotometer at 320 nm ($\epsilon = 8.9\ 10^3$ M^{-1} cm^{-1}) for the free form and at 410 nm ($\epsilon = 1.5\ 10^4$ M^{-1} cm^{-1}) at pH 6.6 for the bound form. Bis-ANS was from Molecular Probes, Junction City, OR. Its concentration was estimated optically at 385 nm, $\epsilon = 16{,}790$ M^{-1} cm^{-1} [9].

2.3 Methods

Terbium (III)-parvalbumin was prepared by the stoichiometric addition of $TbCl_3$ to twice the molar amount of Ca^{2+}–PA. Since lanthanide binding to PA is sequential and since its affinity is much higher than that of Ca^{2+}, only Ca^{2+} bound to the EF-site (EF–Ca^{2+}) was substituted by Tb^{3+} [10,11].

Fluorescence measurements were carried out either on an SLM Model 4800S spectrofluorimeter or a home-made spectrofluorimeter described earlier [12]. All fluorescence spectra were corrected.

The distance, r, between a fluorescent donor and an absorbing acceptor may be estimated from the efficiency of the excitation energy transfer [13]. The "Förster distance", R_0, is defined as the distance, in angstroms, at which 50% energy transfer occurs:

$$R_0 = 9.79\ 10^3 \left(J\kappa^2 Q n^{-4}\right)^{1/6} \tag{1}$$

where J, the overlap integral of the donor emission and the acceptor absorption spectra, is described by the expression

$$J = \int_0^\infty F_d(\lambda)\epsilon_a(\lambda)\lambda^4\, d\lambda$$

in which $F_d(\lambda)$ and $\epsilon_a(\lambda)$ refer, respectively, to the corrected, normalized emission intensity of the donor and the absorption extinction coefficient of the acceptor at wavelength λ. The factor κ^2 describes the relative orientation of the transition dipoles of the donor and the acceptor, Q is the emission quantum yield of the donor, and n is the index of refraction of the medium. For the case of random orientations between the emission and absorption dipoles, the approximation $\kappa^2 = \frac{2}{3}$ may be applied. The refractive index, n, is usually assumed to be 1.33 for protein media [13–15].

The actual distance, r, is then calculated as

$$r = \left[R_0^6(1-E)/E\right]^{1/6} \tag{3}$$

where $E = 1 - F/F_0$ is the energy transfer efficiency and F and F_0 are the fluorescence intensities in the presence and absence of acceptor, respectively.

The course of the reaction of PA with DTNB after mixing of the reagents was monitored by

kinetics of changes in DTNB absorption at 410 nm using a UVICON 860 spectrophotometer.

3. Results and discussion

Figure 1A shows spectrofluorometric titration of Ca^{2+}-loaded cod PA with increasing Cu^{2+} followed by a Co^{2+} titration (Fig. 1B) of the sample in Fig. 1A. It is known that this PA contains one single tryptophan residue per molecule [16] whose fluorescence is extremely sensitive to Ca^{2+}-binding [4,5], where the stoichiometric binding of Ca^{2+} causes a 20 nm blue shift of the fluorescence spectrum concomitant with a 1.5-fold increase in the fluorescence quantum yield. Note that titration of the Ca^{2+}-loaded protein by Cu^{2+} results in a decrease in fluorescence quantum yield (Fig. 1A) without any shift of the fluorescence spectrum (data not shown). These effects appear to be caused in part by specific interactions with the Cu^{2+} ion. The curve in Fig. 1A levels off at $[Cu^{2+}]:[PA] \approx 1$, which suggests strong stoichiometric binding. A subsequent titration of Cu^{2+}, Ca^{2+}–PA by Co^{2+} (or Zn^{2+}) results in a reverse of the Cu^{2+} effect, suggesting that Cu^{2+} and Co^{2+} (Zn^{2+}) compete for the same binding site.

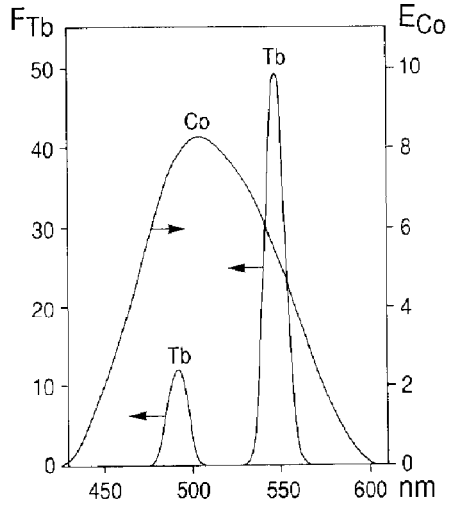

Fig. 2. Normalized emission spectrum of Tb^{3+} (excitation at 374 nm, spectral slit width was 5 nm) and absorption spectrum of Co^{2+} bound to cod PA ($[PA] = 2.4 \times 10^{-4}$ M). $[Tb^{3+}]:[PA] = 1:1$, $[Co^{2+}]:[PA] = 1.5:1$, 10 mM MES, pH 5.6, 25°C. F_{Tb} is relative terbium fluorescence emission; and E_{Co} is cobalt absorbance.

Figure 2 depicts the fluorescence emission spectrum of Tb^{3+} bound to cod PA at pH 5.6 (10 mM MES) [2]. The two prinicipal emission bands

[2] This slightly acidic pH value was chosen in order to avoid the effects of $Tb(OH)_3$ precipitation at higher pH values (exciation wavelength, 374 nm).

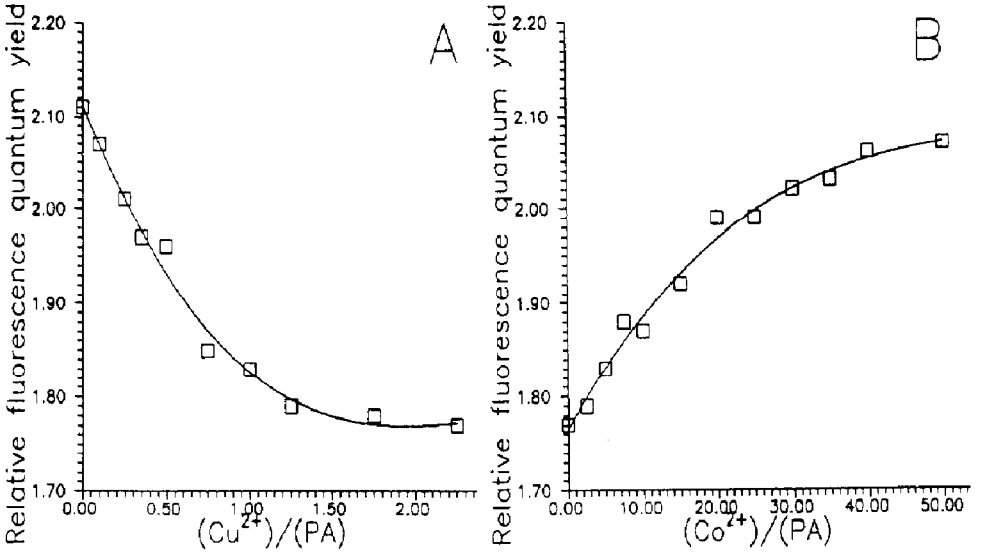

Fig. 1. Spectrofluorimetric titration of Ca^{2+}-loaded cod PA by Cu^{2+} (A) followed by a Co^{2+} titration (B). Protein concentration $[PA] = 2\ 10^{-5}$ M, 10 mM HEPES, pH 7.0, 25°C. The excitation wavelength was 280.4 nm.

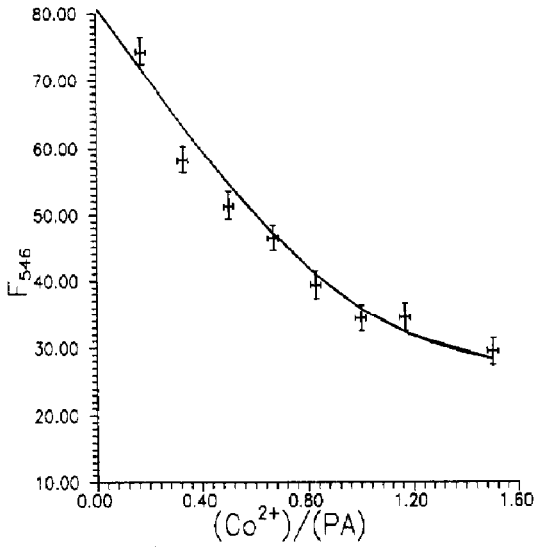

Fig. 3. Cobalt (II) titration of the 1:1 Tb^{3+}–cod PA complex. [PA] = 2.4 10^{-4} M, 10 mM MES, pH 5.6, 25°C. The excitation wavelength was 374 nm. The points are experimental, while the curve is fitted to a one-site binding model.

at 492 and 546 nm are characteristic in this spectrum. Note that these bands overlap well with a broad absorption for bound Co^{2+} shown on the same figure. Figure 3 depicts a cobalt titration of a 1:1 Tb^{3+}–PA complex where a 70% quenching was found for the fully saturated 1:1 complex. The Co^{2+}-binding constant evaluated from the titration curve using the one-site binding scheme was 6 10^4 M^{-1}. The overlap integral was computed graphically from the emission spectrum of the donor and the absorption spectrum of Co^{2+}–PA ($\epsilon_{510\ nm} = 8.2\ M^{-1}\ cm^{-1}$), yielding $J = 4.1\ 10^{-17}\ M^{-1}\ cm^{-3}$.

The value for $K^2 = \frac{2}{3}$ is valid in our case [13]. Firstly, the transition moment of the energy donor, Tb^{3+}, has no preferred direction due to the spherically symmetrical electron outer shell which is not oriented by the ligand field. Secondly, the excitation lifetime of Tb^{3+} is long enough (near 2 ms in aqueous solution) for a randomization of the donor and acceptor orientation by rotational diffusion to occur prior to the energy transfer.

The least certain parameter in these calculations was Q, the quantum yield of the bound Tb^{3+}, since its extremely low extinction coefficient was difficult to accurately measure. We used values of $Q = 0.2$–0.6 that were reported for Tb^{3+} from fluorescence lifetime measurements of inorganic model complexes containing oxygen donors [17]. Using this Q value range for Tb^{3+} we calculated $r = 9.3$–11.2 Å, i.e., a mean Cu^{2+} to EF-Ca^{2+} site distance of 10.3 ± 0.9 Å. This suggests that the mechanical interaction between the Cu^{2+} site and Ca^{2+} site may propagate via the protein structure.

Cod PA possesses three cysteine thiol-groups per molecule [16]. Figure 4 depicts kinetic studies of the reaction of the sulfhydryl reagent DTNB with cod PA thiol-groups at pH 6.6. The binding of Ca^{2+} and Cu^{2+} ions to the protein drastically alters the reactivity to DTNB, and hence thiol group accessibility. Apo-PA displays a high reactivity to DTNB, while the Cu^{2+}, Ca^{2+}-loaded protein shows a minimal rate. We ran suitable control experiments to confirm that Cu^{2+} ions did not hinder the reaction of DTNB with free cysteine. It should be noted that these experiments were designed to examine relative accessibilities, *not* net thiol-group content, hence in the kinetic measurements only a small franction of the total thiol groups have actually reacted.

Figure 5 shows a spectrofluorimetric titration of 1.6 10^{-6} M bis-ANS with increasing cod PA at pH 7.0. In general, a large enhancement in fluorescence emission intensity was observed with

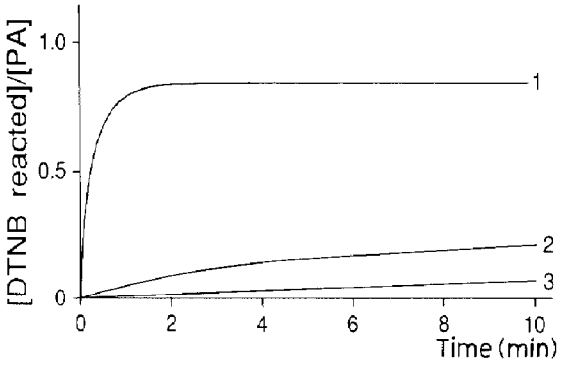

Fig. 4. Kinetics of the reaction of DTNB with different cod PA metal ion states. [PA] = 1.09 10^{-5} M, DTNB = 10 μM (10 mM HEPES, 10 mM MES, pH 6.6), 25°C. The reaction was monitored at 410 nm. 1–apo-PA (0.6 mM EGTA); 2–Ca^{2+}-loaded PA (5 10^{-5} M $CaCl_2$); and 3–Cu^{2+}, Ca^{2+}-loaded PA (5 10^{-5} M $CaCl_2$, 9 10^{-5} M $CuCl_2$).

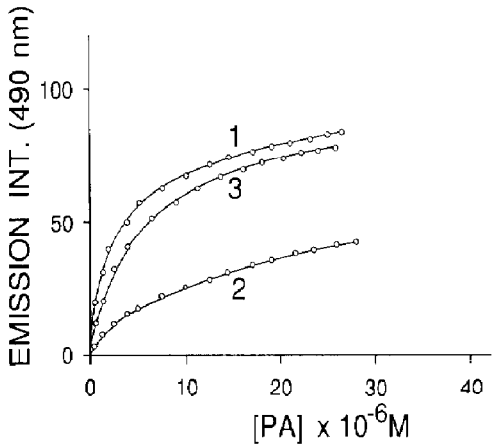

Fig. 5. Spectrofluorimetric titration of 1.6 10^{-6} *M* bis-ANS with increasing cod PA, 10 m*M* HEPES, pH 7.0, 25°C. (1)–apo-protein (1.6 m*M* EGTA); (2)–Ca^{2+}-loaded protein (1 m*M* $CaCl_2$); and (3)–Cu^{2+}, Ca^{2+}-loaded protein (1 m*M* $CaCl_2$, 2 10^{-4} *M* $CuCl_2$). The emission intensity at 490 nm is given in arbitrary units. Excitation wavelength was 385 nm. Curves are the best fits of the data with the one-site binding equation.

increasing protein concentration. The curves in Fig. 5 correspond to three different protein states: Ca^{2+}-loaded; Cu^{2+}, Ca^{2+}-loaded and apo-PA, respectively. Each experimental curve (Fig. 5) was fit to a simple one site binding scheme; the dissociation constants, K_d, are listed in Table 1, which also contains the fluorescence emission enhancement factors and wavelength maxima for each species. From the data, it is clear that apo-PA has the highest affinity for bis-ANS and also yields the largest fluorescence enhancement with Cu^{2+}, Ca^{2+}–PA showing quite similar behavior (Table 1). On the other hand, Cu^{2+}, Ca^{2+}–PA is ca. 8 nm blue-shifted relative to either apo- or Ca^{2+}–PA (Table 1).

Table 1

Equilibrium dissociation constants, K_d, relative fluorescence enhancement factors, EF, and spectral emission maxima, λ_{em}, for complexes of bis-ANS with different metal bound states of cod parvalbumin. (10 m*M* HEPES, pH 7.0, 25°C)

Protein state	K_d (μM)	EF (Rel. units)	λ_{em} (nm)
Ca^{2+}-loaded	5.6±0.5	0.50	490
Cu^{2+}, Ca^{2+}-loaded	3.4±0.5	0.94	482
Apo	2.3±0.5	1.00	490

The results presented above show that cod PA has a distinct Zn^{2+} (Cu^{2+}) binding site located ca. 10.3 ± 0.9 Å from the Ca^{2+}-binding site. The physical conformation of Cu^{2+}, Ca^{2+}-loaded PA is different from Ca^{2+}-PA. The binding of Cu^{2+} decreases protein thermostability [6], increases its affinity to bis-ANS and decreases its reactivity to DTNB [3]. This behavior is reminiscent of quite similar results with α-lactalbumin, since the binding of Cu^{2+} or Zn^{2+} to α-lactalbumin increases its affinity for bis-ANS [18] and decreases its thermostability [7]. The binding of Cu^{2+} or Zn^{2+} to either Ca^{2+}–PA or Ca^{2+}–α-lactalbumin converts them to an "apo-like" state, although this effect is more pronounced in the case of α-lactalbumin. In contrast to calmodulin, which is more hydrophobic in the Ca^{2+}-loaded state, both PA and α-lactalbumin possess hydrophobic surfaces which are more exposed to hydrophobic probes (such as bis-ANS), in the apo- (or Cu^{2+}, Ca^{2+})-state. It is also interesting to note that apo-PA is able to bind small polypeptides, such as melittin [7], yet the binding of Ca^{2+} inhibits this interaction.

The general observation that many Ca^{2+}-binding proteins (e.g., parvalbumin, α-lactalbumin, calmodulin, and S-100 protein) possess distinct separate Zn^{2+}-binding sites may be of profound physiological importance. It suggests that Zn^{2+} ions may play an important regulatory role in biological systems, the specifics of which we do not presently understand.

Acknowledgement

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[3] Preliminary X-band EPR spectra of a Cu^{2+}, Ca^{2+}–PA complex in 10 m*M* HEPES, pH 6.8, 77 K, were devoid of superhyperfine splittings in the $g_\perp$ region of the spectrum (i.e., $g \approx 2.0$). Note that cod PA contains no histidine residues.

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