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Fluorescence studies on the Ca^{2+} and Zn^{2+} binding properties of the α-subunit of bovine brain S-100a protein

Iris K.M. Leung, Rajam S. Mani and Cyril M. Kay

Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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The single cysteine on the α-subunit of bovine brain S-100a protein has been modified with the thiol specific probe, Acrylodan. When the labelled apoprotein was excited at 380 nm the fluorescence emission maximum was centered at 484 ± 2 nm, suggesting that the probe is in a fairly hydrophobic environment. Addition of Ca^{2+} to the protein caused the emission maximum to undergo a red shift to 504 ± 2 nm, implying that the fluorophore is now more exposed to the solvent. Zn^{2+}, when added to the protein, induced only a small perturbation and the emission maximum shifted to 481 ± 2 nm. Ca^{2+} was able to perturb the fluorophore in the presence of Zn^{2+}. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS)-labelled α-subunit when excited at 345 nm exhibited very little fluorescence in the absence of Ca^{2+}. Addition of Ca^{2+} resulted in an increase in TNS fluorescence accompanied by a blue shift of the emission maximum to 445 ± 1 nm indicating that the probe in the presence of Ca^{2+} moves to a hydrophobic domain. The fact that Ca^{2+} and Zn^{2+} can perturb the labelled sulfhydryl group in the presence of each other clearly demonstrates that the binding sites for the two metal ions must be different on the α-subunit as well as on the S-100a protein.

S-100 protein; Ca^{2+} effect; Zn^{2+} effect; Fluorescence

1. INTRODUCTION

Bovine brain S-100 protein is actually a mixture of two major components, S-100a and S-100b, with a subunit composition of $\alpha\beta$ and β_2, respectively [1]. Each of the subunits contains a functional Ca^{2+}-binding loop at the C-terminus, termed a EF hand, based on the crystal structure of parvalbumin [2] and both subunits bind Ca^{2+} with similar affinity [3–5]. The α-subunit in S-100a is characterized by the presence of a single tryptophan (residue 90) and a single cysteine (residue 85) positioned in close proximity in the C-terminal region of the molecule. The possible contribution of the state of sulfhydryl groups of the S-100 proteins to their electrophoretic heterogeneity, conformational state and reactivity towards specific antibodies has already been reported [6–9]. In order to examine the nature of the change in the environment of the sulfhydryl groups upon Ca^{2+} and Zn^{2+} binding to α-subunit, we have used the thiol specific, polarity sensitive fluorescent probe Acrylodan to label the protein, α-subunit is an ideal system for this purpose, since the protein has only 1 cysteine at position 85.

Correspondence address: I.K.M. Leung, Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Abbreviations: Mops, (3-[*N*-morpholino]propanesulfonic acid); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; CD, circular dichroism

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2. MATERIALS AND METHODS

S-100a protein and the α-subunit were prepared from bovine brain by using the methodology described in an earlier paper [10]. Protein concentration was determined using $E^{1\%}_{1cm,278nm}$ values of 7.98 and 5.4 for the α-subunit and S-100a, respectively. Acrylodan and TNS sodium salt were purchased from Molecular Probes (Junction City, OR) and used without further purification. The sulfhydryl group in the α-subunit was titrated by the method of Ellman [11] as described in [12].

2.1. *Preparation of Acrylodan-labelled α-subunit and S-100a protein*

The proteins were labelled following the procedure of Prendergast et al. [13]. Acrylodan was first dissolved in dimethylformamide. The α-subunit in 50 mM Mops (pH 7.0) was incubated with excess Acrylodan at 4°C for 12–14 h. After the labelling, the proteins were subjected to clarification by centrifugation. The proteins were then dialysed against the starting buffer with 4 mM EDTA added and thereafter exhaustively dialysed versus 0.1 M Tris-HCl buffer (pH 7.5), which had been passed through a Chelex-100 column. Protein concentrations were established in the ultracentrifuge by employing a Rayleigh interference optical system, assuming 41 fringes equivalent to 10 mg/ml [14]. The concentration of the label was determined from the absorbance at 360 nm using a molar extinction coefficient of 12900 $M^{-1} \cdot cm^{-1}$ [13].

2.2. *TNS labelling*

The fluorescent probe, TNS, was dissolved in ultrapure water (Milli-Q Instrument, Millipore-Waters Corp.) just before use. Fluorescence titrations were performed by adding aliquots of stock TNS solution to the protein. The concentration of stock TNS solution was determined from the absorbance at 317 nm assuming a molar extinction co-efficient of 1.89×10^4 M [15]. All fluorescence measurements were carried out in a Perkin-Elmer MPF-44B spectrofluorometer as outlined in [16]. CD measurements were performed in a Jasco J500C spectropolarimeter fitted with a DP-500N processor as described [17].

3. RESULTS

3.1. *Reaction of α-subunit with DTNB*

The single sulfhydryl group at position 85 on the α-subunit was essentially inaccessible in 1 mM EDTA but became exposed upon denaturation in 6 M Gdn-HCl. In the presence of Zn^{2+}, the rate of reaction with DTNB was slow, and after 1 h, only 0.2 thiol group/chain reacted whereas in the presence of Ca^{2+}, 0.65 SH groups was able to interact with DTNB. Hence the single sulfhydryl group is more exposed to the solvent in the presence of Ca^{2+}.

3.2. *Interaction of Acrylodan with the α-subunit and S-100a protein*

Labelling of the α-subunit with Acrylodan in the presence of Ca^{2+} resulted in labelling 0.31 thiol group/α-chain. In the case of S-100a where there are three sulfhydryls, DTNB studies revealed that one of these was reactive in the case of the apoprotein as opposed to two thiols being titrated in the presence of Ca^{2+} [12]. Therefore, in order to maintain a fluorophore:protein labelling ratio of less than 1 as observed with the α-subunit, S-100a was reacted with Acrylodan in the presence of 1 mM EDTA. The number of sulfhydryl groups labelled was 0.35.

The fluorescence spectra of the Acrylodan-labelled α-subunit are shown in fig.1. When the apoprotein was excited at 380 nm, the wavelength of the emission maximum occurred at 484 ± 2 nm. In the presence of Ca^{2+}, at pH 7.5, the emission maximum of the protein underwent a red shift to 504 ± 2 nm with no significant increase in the fluorescence intensity. When the protein-Acrylodan adduct was dissolved in 6 M Gdn-HCl, the fluorescence λ_{max} shifted to 525 ± 2 nm. This implies that the fluorophore, when fully exposed to the solvent, has a λ_{max} around 525 ± 2 nm. Hence, it would appear that the 6-acryl-2-dimethylaminonaphthalene moiety attached to the single thiol group in the apoprotein is in a fairly hydrophobic environment. The red shift observed upon Ca^{2+} addition to the α-subunit is due to a conformational change induced by metal ion binding whereby the probe moves to a more polar microenvironment. Addition of Zn^{2+} to the labelled apoprotein had no significant effect on either the wavelength of the emission maximum (481 ±

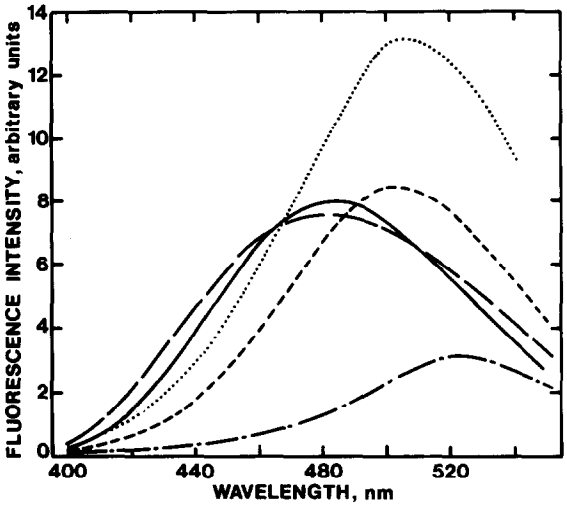

Fig.1. Fluorescence emission spectra of the α-subunit of S-100a protein at 25°C, in 0.1 M Tris-HCl buffer, pH 7.5, free of divalent cations (——); in 0.1 M Tris-HCl buffer, pH 7.5, containing 1.09 mM Ca^{2+} (---); in 0.1 M Tris-HCl, pH 7.5, containing 0.4 mM Zn^{2+} (— —); in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.4 mM Zn^{2+} and 1.08 mM Ca^{2+} (···); and in 6 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.5 (—·—).

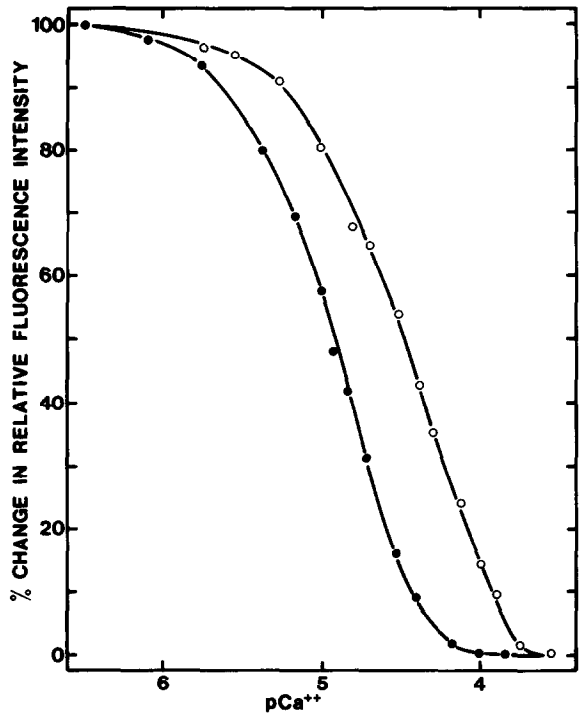

Fig.2. Percentage change in the fluorescence intensity at 450 nm as a function of pCa^{2+} for the α-subunit of S-100a protein in 0.1 M Tris-HCl buffer, pH 7.5 (●) and in 0.1 M Tris-HCl buffer, pH 7.5, containing 90 mM KCl, (○) at 25°C. The concentration of the protein was 1.19×10^{-5} M.

2 nm) or the fluorescence intensity, suggesting that Zn^{2+} was not altering to any extent the conformation around the single thiol group and for this reason the labelled protein was titrated only with calcium. The titration curve generated by monitoring the fluorescence intensity at 450 nm as a function of Ca^{2+} concentration is shown in fig.2, from which a K_d value of 1.2×10^{-5} M was derived and this corresponds to the Ca^{2+} concentration required to induce a 50% change in the observed fluorescence intensity. In the presence of 90 mM KCl, the protein binds Ca^{2+} with lower affinity (K_d, 3.31×10^{-5} M) implying that KCl acts as an antagonist towards Ca^{2+}, in agreement with our earlier fiinding [10]. However, if Zn^{2+} was added to the α-subunit after the addition of Ca^{2+}, it was able to produce a further increase in the fluorescence intensity (not shown) suggesting that Zn^{2+} can bind and induce a further conformational change in the Ca^{2+} bound protein. Similarly, Ca^{2+} was able to induce a conformational change in the presence of Zn^{2+}. It is obvious from these two experiments that Ca^{2+} and Zn^{2+} can bind and perturb the thiol group in the presence of each other and is consistent with our recent findings with the β-subunit [18].

The fluorescence spectra of S-100a are shown in fig.3. Addition of Zn^{2+} to labelled S-100a caused the emission maximum to undergo a blue shift, from 480 ± 2 nm to 470 ± 2 nm. In the presence of Ca^{2+} or 6 M Gdn-HCl, the emission maximum occurred at 491 ± 2 nm and 525 ± 2 nm, respectively. The observed blue shift suggests that Zn^{2+} induced the fluorophore to move to a less polar environment whereas the effect of Ca^{2+} was opposite. The results clearly reveal that the probe occupies different environments depending on the nature of the metal ion bound. Incorporation of the label into α-subunit and S-100a proteins did not result in any significant alteration in the secondary structure of these proteins and this was checked by far UV CD measurements.

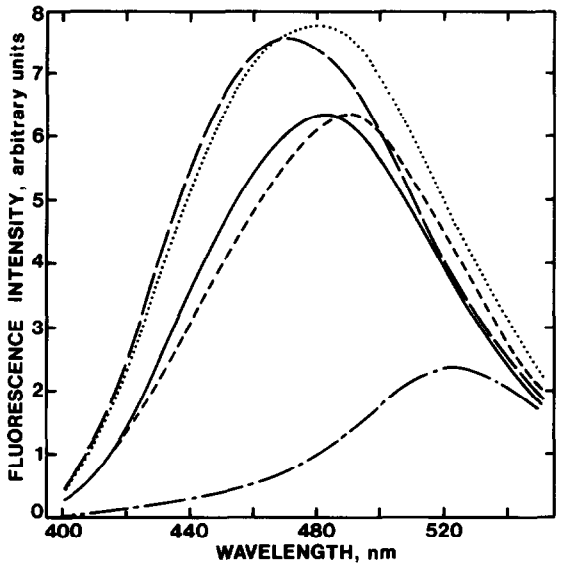

Fig.3. Fluorescence emission spectra of S-100a protein at 25°C, in 0.1 M Tris-HCl buffer, pH 7.5, free of divalent cations (——); in 0.1 M Tris-HCl buffer, pH 7.5, containing 1.09 mM Ca^{2+} (---); in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.41 mM Zn^{2+} (— —); in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.41 mM Zn^{2+} and 1.09 mM Ca^{2+} (···); and in 6 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.5 (—·—).

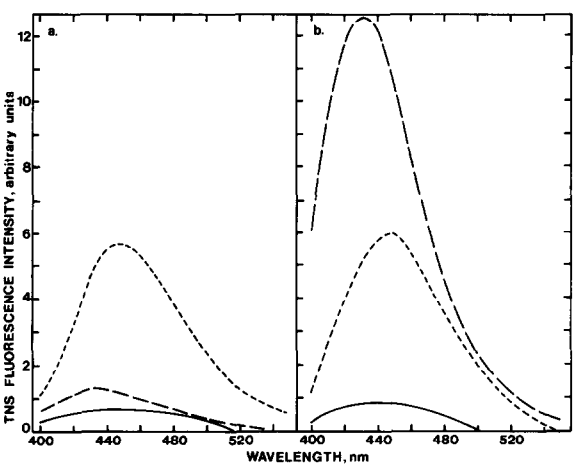

Fig.4. Fluorescence spectra of TNS bound to (a) α-subunit (5.3×10^{-6} M in 0.1 M Tris-HCl buffer, pH 7.5, with 15.1 μM TNS) and (b) S-100a protein (5.3×10^{-6} M in 0.1 M Tris-HCl buffer, pH 7.5, with 15.1 μM TNS) under various conditions; in the absence of any divalent cations (——); in the presence of 1 mM Ca^{2+} (---); in the presence of 0.4 mM Zn^{2+} (— —).

3.3. *Interaction of TNS with the α-subunit and S-100a probe*

It has been postulated that binding of Ca^{2+} to a mixture of S-100 protein induced a conformational change involving the partial exposure of a hydrophobic domain [19]. The hydrophobic probe, TNS, fluoresces weakly in polar solvents but its intensity becomes enhanced in apolar solvents or when bound to a hydrophobic pocket on the protein [15]. As seen in fig.4a and b, the fluorescence of TNS in the presence of metal free α-subunit and S-100a was negligible. Addition of Ca^{2+} to the α-subunit and S-100a produced a 9- and 7-fold enhancement in TNS fluorescence, respectively, accompanied by a blue shift of the emission maximum in both proteins to 445 ± 1 nm, implying that the probe now occupies a more hydrophobic environment. Addition of 0.4 mM Zn^{2+} induced a large blue shift with the emission maximum occurring at 430 ± 1 nm for both the α-subunit and S-100a protein. However, with the former, the TNS fluorescence did not change significantly, whereas a 14-fold enhancement was observed for the latter. The TNS probe could bind either at the N-terminal hydrophobic region (residues 4–14) or at the C-terminal end involving residues 73–80 based on the amino acid sequence data of the α- and β-subunit [1]. Histidine residues which are known to act as ligands for coordinating Zn^{2+} [22] are only found at the N-terminus of the α-subunit at position 16 and 18 and since Zn^{2+} addition did not result in any increase in fluorescence intensity of the TNS-labelled protein, we believe that the probe is located at the C-terminal end, well away from the histidine residues. In the case of S-100a, TNS could bind at either or both of the two hydrophobic regions on the β-subunit, in addition to the C-terminal end of the α-chain, resulting in the observed increase in fluorescence intensity.

4. DISCUSSION

The isolation of the α-subunit of bovine brain S-100a protein and the characterization of its metal ion binding properties have been reported by two laboratories [10,20]. A comparison of the far and near UV CD and fluorescence results reported by these laboratories suggests that the two preparations of the α-subunit are similar. This is also true

with the parent S-100a protein [16,21] from which the α-subunit was isolated. However, it should be noted that our S-100a and the α-subunit differ from the corresponding proteins of Baudier and Gérard [21] only in the absolute $\Delta\epsilon$ values determined by UV difference spectroscopy. Nevertheless, the effect of Ca^{2+} on both proteins from the two laboratories is the same, namely, upon metal addition there is an exposure of the aromatic groups, tryptophan and tyrosines to solvent, i.e. a blue shift was observed. Hence, S-100a and the α-subunit isolated and studied by both groups represent the same protein species.

Addition of Ca^{2+} to Acrylodan-labelled α-subunit resulted in exposing the probe to solvent. Since earlier spectral studies suggested that the single tryptophan in the protein moved to a more polar environment in the presence of Ca^{2+} [10], we believe that the Ca^{2+} effect is localized in the C-terminus, where Tyr-74, Cys-85, Phe-88, -89 and Trp-90 are located. On the contrary, Zn^{2+} induced only a small perturbation of the fluorophore, implying that its binding site is not in close proximity to cysteine 85. The small perturbation observed with Zn^{2+} may be attributed to the fact that histidine residues, which act as ligands for coordinating Zn^{2+} [22] are only found at positions 16 and 18 well away from cysteine 85 located at the C-terminal end.

Of the three sulfhydryl groups in S-100a protein ($\alpha\beta$), two are located at position 68 and 84 on the β-chain and the third sulfhydryl group is present in the α-subunit at position 85. According to Nika et al. [23], the reactive thiol occurred at position 84 on the β-chain. Therefore, the single thiol group that reacted with the 6-acyl-2-dimethylaminonaphthalene moiety under native solvent conditions probably corresponds to cysteine 84 on the β-chain. Upon comparing the metal ions binding properties of the α-subunit and S-100a protein, it can be seen that Ca^{2+} exerts a similar effect on the microenvironment near the probe in both proteins, namely the exposure of the fluorophore to solvent. In contradistinction, the two proteins do not respond to Zn^{2+} binding in a similar manner. In the case of S-100a, probable Zn^{2+} binding to additional histidines at the C-terminus of the β-chain could explain the observed larger effect on S-100a protein compared to the α-subunit.

In conclusion, using the thiol specific probe, Acrylodan, we have demonstrated that Ca^{2+} induced a conformational change in the C-terminus of the α-subunit and S-100a protein. Zn^{2+} induced only a small perturbation in the α-subunit, suggesting that its binding site is not in close proximity to the labelled cysteine at the C-terminus. The fact that Ca^{2+} and Zn^{2+} can perturb the labelled sulfhydryl group in the presence of each other clearly demonstrates that the two metal ion binding sites must be different on the α-subunit as well as on the S-100a protein.

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