A fluorimetry study of \( N\)-(1-pyrenyl)iodoacetamide-labelled bovine brain S-100a protein

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S-100 a protein has been modified with the thiol specific fluorescent label \( N\)-(1-pyrenyl)iodoacetamide. When the protein was reacted with the fluorophore in the presence of EDTA, only one sulphydryl group was labelled. The labelled protein when excited at 344 nm had an emission maximum at 386 nm and a shoulder at 403 nm. Addition of calcium resulted in a quenching of the fluorescence intensity at 386 nm whereas \( Zn^{2+} \) addition caused an enhancement. Titration of labelled protein with \( CaCl_2 \) or \( ZnSO_4 \) yielded dissociation constants \( (K_D) \) values of \( 3 \times 10^{-5} \) M and \( 8 \times 10^{-6} \) M for \( Ca^{2+} \) and \( Zn^{2+} \), respectively. In the presence of 90 mM KCl the protein has a lower affinity towards calcium \( (K_D, 6 \times 10^{-5} \) M). Acrylamide quenching studies revealed the probe to be more accessible to acrylamide in the plus calcium state. Reaction kinetics with DTNB in 0.1 M Tris buffer at pH 7.5 also suggested that the single reactive sulphydryl group was more exposed to the solvent in the presence of \( Ca^{2+} \). Thus, the observed quenching of calcium addition may be the result of the probe moving to a relatively more polar environment. This would imply that the probe moves to a more nonpolar region in the presence of \( Zn^{2+} \). These results are in accord with our earlier near UV-CD studies where the metal ions, calcium and zinc were observed to induce different conformational changes in S-100a protein.

1. INTRODUCTION

The highly acidic water-soluble S-100 protein [1] is considered mainly a nervous tissue-specific protein found primarily in the cytoplasm of glial cells. Recently, Gayner et al. [2] have shown the presence of S-100 protein in continuous cell lines of human malignant melanoma. The biological function of this protein is unknown, but the fact that it is found in the brain of both invertebrates and vertebrates with a constant immunological response [3] indicates that this protein has been conserved throughout evolution and hence may have an important role in the functioning of the nervous system.

S-100 protein is actually a mixture of two components, S-100a and S-100b, with a subunit composition of \( \alpha \beta \) and \( \beta_2 \), respectively [4]. The amino acid sequences of S-100 proteins have been determined [4,5]. The S-100b protein polypeptide chain (\( \beta \)-subunit) consists of 91 amino acid residues and its sequence is similar to calcium binding proteins such as calmodulin [6,7], troponin-C [8] and parvalbumin [9]. The \( \alpha \)-subunit in S-100a consists of 93 amino acid residues, possesses an extensive sequence homology (58%) with that of the \( \beta \)-subunit but 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 [4]. According to
Isobe and Okuyama this region is preceded by the putative Ca$^{2+}$ binding site on the basis of the EF-hand model [10]. A Ca$^{2+}$ binding site of this type was also predicted for the $\beta$-subunit and within this site is one of the two sulfhydryls in the $\beta$-chain (position 68). The additional sulfhydryl group (residue 84) contained within the subunit is present 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 [11-14]. S-100 proteins undergo a conformational change upon binding calcium ion [15-20]. In the case of S-100a, Ca$^{2+}$ binding induces a restricted conformational change in the protein resulting in the exposure of the single tryptophan and one or more phenylalanine and tyrosine residues.

Here, using the sulfhydryl fluorescent probe PIA, we have demonstrated that addition of Ca$^{2+}$ induced changes in the microenvironment around the labelled sulfhydryl group. In addition, acrylamide quenching studies were carried out with the labelled protein, in the presence and absence of calcium. Results obtained from these studies clearly demonstrate that the single sulfhydryl group that is labelled moves to a more polar environment in the presence of Ca$^{2+}$.

### 2. MATERIALS AND METHODS

S-100a protein was prepared from bovine brain using the methodology described in earlier publications [17,18]. Protein concentration was determined using an $E_{1cm}^{1%}$ of 5.4. A Perkin-Elmer model MPF-44B recording spectrofluorimeter was used for fluorescence measurements as described in [18]. The protein was initially dissolved in appropriate buffer in the presence of EDTA and, thereafter, was subjected to exhaustive dialysis with at least four changes against the solvent that had been passed through the Chelex-100 columns.

#### 2.1. Preparation of pyrene-S-100a protein

PIA was purchased from Molecular Probes (USA) and used without further purification. PIA was first dissolved in dimethylformamide. The protein in appropriate solvent was incubated with PIA for 16-24 h in the dark. After incubation the protein was subjected to clarification in a Beckman Model L8-70M centrifuge. After centrifugation the clear supernatant was subjected to exhaustive dialysis with the solvent that had been passed through a Chelex-100 column. The protein concentration of pyrene-S-100a was measured using the Bradford [21] protein assay with native S-100a as the standard. The concentration of label was determined from the absorbance at 344 nm using an extinction coefficient of 22000 M$^{-1}$ cm$^{-1}$ [22].

#### 2.2. Sulfhydryl titration with DTNB

The three sulfhydryl groups in S-100a were titrated by the method of Ellman [23]. The number of sulfhydryl group derivatized/mol of S-100a was calculated, assuming an extinction coefficient of 13600 mol$^{-1}$ for the liberated 5-nitro-2-thiobenzoate and an $M_r$ of 21000 for the S-100a protein.

#### 2.3. Acrylamide fluorescence quenching studies

The excitation wavelength used was 344 nm, at which the probe has the maximum absorption. In all instances the initial $A_{344}$ of the protein solution was ≤0.05; thus avoidance of the inner-filter effect was assured. The fluorescence quenching was measured at the emission maximum (386 nm) of the protein and was initiated by adding small aliquots (10 µl) of 8 M acrylamide solution. Cells of 1 cm pathlength were employed and stirring was by a magnetic ‘flea’. Protein solutions of 2 ml were used. The theory of acrylamide quenching and the mathematical treatment of the data are discussed in [24].

#### 2.4. CD measurements

Circular dichroism measurements were made on a Jasco J500C instrument fitted with a DP500N data process unit. The instrument was routinely standardized with d-10-camphorsulfonic acid and pantoyl lactone.

### 3. RESULTS

#### 3.1. Reaction of S-100a with DTNB

The rate of reaction of S-100a protein with DTNB under different experimental conditions is depicted in fig.1. Our S-100a protein preparation showed a significant increase in reactivity towards DTNB in the presence of 1 mM Ca$^{2+}$, amounting to 1.2 thiol groups reacting per S-100 molecule.
Fig. 1. Rate and extent of reaction of S-100a with DTNB. (1) in 1 mM EDTA; (2) in 1 mM free Ca$^{2+}$, and (3) in 6 M Gdn-HCl. The solvent was 0.1 M Tris--HCl buffer (pH 7.5) and the protein concentrations were 0.32 mg/ml in 1 mM EDTA, 0.14 mg/ml in the 1 mM Ca$^{2+}$ case and 0.15 mg/ml in the 6 M Gdn-HCl medium.

Similar observations have been made with S-100 proteins by Dannies and Levine [12] and Nika et al. [25]. In the presence of 1 mM EDTA, the rate of reaction was very slow and after about 60 min only 0.8 thiol group had reacted. However, when the protein was denatured in 6 M Gdn-HCl, 2.7 thiol groups were readily available to the reagent, suggesting that under this condition all three sulfhydryl groups reacted with DTNB. From these experiments it is obvious that under native solvent conditions only one sulfhydryl group in S-100a is reactive and this group becomes more exposed to the solvent in the presence of calcium.

3.2. Interaction of PIA with S-100a

PIA has been used by several workers specifically to label sulfhydryl groups of actin and the labelled actin was fully capable of undergoing polymerization [22, 26]. In order to investigate the reactivity of sulfhydryl groups of S-100a towards PIA, S-100a was incubated with excess dye in the presence of 0.1 M Tris buffer (pH 7.5) and 1 mM EDTA. Under these experimental conditions only one sulfhydryl group in S-100a was labelled.

The fluorescence spectra of S-100a are presented in fig. 2. With excitation at 344 nm, the fluorescence spectrum of S-100a is characterized by a peak at 386 nm and a shoulder around 403 nm. Addition of calcium at pH 7.5 caused an approx. 35% decrease in fluorescence intensity with no significant change in the position of the emission maximum. When calcium was added to S-100a in the presence of 90 mM KCl, the observed quenching in relative fluorescence intensity at 386 nm was even greater (~40%). Addition of Zn$^{2+}$ to labelled S-100a caused an increase in fluorescence intensity (fig. 2) and the magnitude of the increase was nearly 15% with no change in the position of the emission maximum. This observation clearly reveals the fact that the probe on the protein occupies different environments depending on the metal ion (Ca$^{2+}$ or Zn$^{2+}$) that is present. Since the observed quenching in fluorescence intensity with calcium addition (at 386 nm) was nearly 35%, a calcium titration was carried out with the labelled protein. A plot of the change in fluorescence intensity at 386 nm vs calcium added is indicated in fig. 3, and from the fluorescence titration data, the dissociation constant for calcium ($K_D$) was estimated to be $3 \times 10^{-5}$ M and decreased ($K_D$, $6 \times 10^{-5}$ M) when the solvent system used included 90 mM KCl. Thus, the protein has a lower affinity for calcium in the presence of KCl and this finding is consistent with earlier observations [18]. A value of $8 \times 10^{-6}$ M was determined as the dissociation constant ($K_D$) for Zn$^{2+}$ (fig. 4). The protein seems to have a higher affinity for Zn$^{2+}$ (by an order of magnitude) com-
pared with Ca\(^{2+}\); the physiological significance of this is not clear as yet since the precise biological function is not known with certainty.

In order to ensure that the label has not caused any major structural change in the protein, control CD experiments were carried out in the far UV region with the labelled S-100a. This is particularly important since there is no known bioassay system for this protein. When the protein was dissolved in 6 M Gdn-HCl Tris buffer (pH 7.5) and reacted with PIA, all three sulfhydryl groups in S-100a were labelled. Addition of Ca\(^{2+}\) to calcium-free S-100a labelled protein produced less than 10% quenching in fluorescence intensity at 386 nm. Control CD experiments revealed major structural alteration in the protein. Thus, the observed effect of calcium, i.e., less than 10% quenching as opposed to nearly 35% with the native labelled protein, could be a consequence of the induced structural change in the protein or, alternatively, when all the 3 sulfhydryl groups are labelled, the additional two labelled groups respond differently towards Ca\(^{2+}\), and what we observe here is probably the net resultant effect of Ca\(^{2+}\) on S-100a.

### 3.3. Acrylamide quenching

Fig. 5 shows the effect of the acrylamide quencher on the fluorescence intensity of PIA in S-100a. The linear portion of the plot at low acrylamide concentration gave Stern-Volmer constants (\(K_{SV}\)) of 0.50 M\(^{-1}\) for PIA-S-100a in 1 mM EDTA and a value of 0.76 M\(^{-1}\) for the labelled protein in the presence of 1 mM Ca\(^{2+}\). In the presence of 0.5 mM Zn\(^{2+}\), the \(K_{SV}\) value obtained was 0.25 M\(^{-1}\). The effect of including 90 mM KCl in the solvent system is also shown in fig. 5. From the slope, \(K_{SV}\) values of 0.58 M\(^{-1}\) and 1.0 M\(^{-1}\) were determined for the labelled protein in 1 mM EDTA and 1 mM Ca\(^{2+}\), respectively. The values of the Stern Volmer constants obtained suggest that the probe is more accessible to acrylamide in the presence of Ca\(^{2+}\) and the inclusion of KCl in the solvent makes the probe even more accessible to acrylamide. At high acrylamide concentration the effect of quenching seems to level off (fig. 5), and this may be considered as an example of downward
curvature from linearity due to reduced efficiency of quenching at high acrylamide concentration. This downward curvature has also been observed with other protein systems [27].

4. DISCUSSION

Interaction of bovine brain S-100 proteins with Ca$^{2+}$ has been studied by several workers [15,16,25,28]. Upon binding Ca$^{2+}$, S-100a protein undergoes a conformational change resulting in the exposure of its single tryptophan and one or more phenylalanine and tyrosine residues. Also, sulfhydryl groups have been implicated in the calcium binding process [15,25] based on the appearance of multiple bands during electrophoresis and the reactivity of the protein with specific antibodies [11–13]. S-100a has three sulfhydryl groups and only one sulfhydryl group is present in the $\alpha$-chain (residue 85) and the other two are present in the $\beta$-chain in positions 68 and 84. All three sulfhydryls in S-100a reacted with DTNB when the protein was initially denatured in 6M Gdn-HCl (fig.1). From the figure, it is also apparent that the single sulfhydryl group which reacts with DTNB under native conditions becomes more exposed to solvent in the presence of Ca$^{2+}$. Of the three sulfhydryl groups in S-100a, the reactive thiol group occurs in the C-terminal portion (residue 84) of the $\beta$-chain and is characterized by a cluster of hydrophobic amino acid residues. $[^{14}\text{C}]$NEM labelling and peptide analyses by HPLC revealed this thiol group to have most of the label [25]. According to Nika et al. [25] the other thiol group present in the $\beta$-chain in position 68 was shielded in the intact protein even in the presence of Ca$^{2+}$. The only thiol group (residue 85) in the $\alpha$-chain present in the C-terminal end had labelling only when the protein was denatured, suggesting that this thiol is also not readily available for labelling. Hence, the single thiol group that is labelled under benign solvent condition probably corresponds to the thiol group (residue 84) present in the C-terminal region of the $\beta$-chain.

PIA has been used in recent years as a fluorescent probe to label thiol groups in proteins. The probe has an absorption maximum around 344 nm and when excited at 344 nm it usually has an emission maximum for the monomer centered around 385 nm and a shoulder at 403 nm. Sometimes, the probe also exhibits an emission peak at 485 nm and this results from the formation of excited dimers (i.e., excimers) of pyrene [29]. The excimer is indicative of the proximity of the labelled cysteine residues in a protein. When S-100a was labelled with PIA in the presence of 1mM EDTA, only one SH-group was labelled and, as discussed earlier, we believe it is the sulfhydryl group (residue 84) that is present near the C-terminal end of the $\beta$-chain. When excited at 344 nm, the emission maximum occurred at 386 nm with a shoulder around 403 nm. However, there was no evidence of excimer fluorescence and so the two SH-groups near the C-terminal end of the $\alpha$ and $\beta$ chains are not close enough to produce excited dimers.

Addition of Ca$^{2+}$ to labelled S-100a resulted in quenching the fluorescence intensity at 386 nm by almost 35% and we believe this quenching occurs as the probe gets more exposed to the solvent in the presence of Ca$^{2+}$. This conclusion is based on the observation that reaction with DTNB is much faster in the presence of Ca$^{2+}$. More direct evidence comes from acrylamide quenching studies where the probe was more accessible to acrylamide in the presence of Ca$^{2+}$. The Stern-Volmer constants obtained, $K_{SV}$ values, are a measure of this property and the observed values in the presence and absence of Ca$^{2+}$ were 0.76 and 0.5 M$^{-1}$, respectively. When Ca$^{2+}$ and Zn$^{2+}$ titrations were carried out with the labelled protein at pH 7.5, the curve exhibited one inflection point with a dissociation constant, $K_D$, of $3 \times 10^{-5}$ M for Ca$^{2+}$ and $8 \times 10^{-6}$ M for Zn$^{2+}$. Both the latter values are within the physiological concentration of these cations in brain. Inclusion of 90mM KCl in the solvent system (a physiologically relevant concentration in brain) resulted in lowering the affinity for calcium and this is consistent with earlier findings on this system [18]. However, the $K_D$ value of $3 \times 10^{-5}$ M obtained for Ca$^{2+}$ using the probe is slightly lower compared with the $K_D$ value of $1.2 \times 10^{-4}$ M estimated using the protein's intrinsic fluorescence measurements. In other words, in the presence of the probe the protein has, in fact, a higher affinity towards Ca$^{2+}$ by nearly half an order of magnitude. Addition of Zn$^{2+}$ to labelled protein resulted in an increase in fluorescence intensity and this could mean the probe moves to a hydrophobic pocket. This is consistent with the acrylamide quenching studies, since the Stern-
Volmer constant obtained was only 0.25. Hence, the probe is least accessible to acrylamide in the presence of Zn²⁺ (fig.5), whereas in the presence of Ca²⁺ the probe moves to a more polar environment. Near UV-CD studies [19] have also established that Zn²⁺ and Ca²⁺ have different effects on the aromatic chromophores of S-100a. From the results obtained with the experimental protocol used here, it is apparent that PIA is a good microenvironmental probe with fairly sensitive fluorescence intensity, but with insensitive shift of the emission maximum. For the first time, using a thiol specific fluorescent probe on a single reactive sulfhydryl group in this system, we have demonstrated that individual addition of the metal ions Ca²⁺ and Zn²⁺ perturbs the sulfhydryl environment in S-100a differently.

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