Isolation, characterization and metal-ion-binding properties of the α -subunit from S-100a protein

Iris K. M. LEUNG, Rajam S. MANI and Cyril M. KAY*

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

The brain-specific S-100 protein is a mixture of two predominant components, S-100a and S-100b, with subunit compositions of $\alpha\beta$ and $\beta\beta$ respectively. In the present study, the α -subunit, isolated from S-100a by using anion-exchange chromatography in the presence of 8 M-urea, was homogeneous by the criteria of SDS/polyacrylamide-gel, urea/SDS/polyacrylamide-gel and non-SDS/polyacrylamide-gel electrophoresis. The α -subunit underwent a conformational change upon binding Ca^{2+} and Zn^{2+} at pH 7.5, as revealed by u.v. difference spectroscopy, c.d. and fluorescence measurements. Far-u.v. c.d. studies indicated the apparent α -helical content to fall when the protein bound either Ca^{2+} or Zn^{2+} . Addition of Ca^{2+} to the α -subunit resulted in exposing to the solvent the single tryptophan residue and one or more tyrosine and phenylalanine residues. Zn^{2+} induced only a small conformational change, and among the aromatic chromophores only tyrosine residues were affected to a small extent. Ca^{2+} was able to bind to the α -subunit in the presence of Zn^{2+} , and the two metal-ion-binding sites appeared to be different. When the apoprotein was excited at 280 nm, the fluorescence emission maximum was located at 337 nm. In the presence of Ca^{2+} , the emission maximum occurred at 340 nm and was accompanied by a nearly 25% increase in fluorescence intensity. Fluorescence titration with Ca^{2+} at pH 7.5 revealed only one class of binding site, with a K_d value of 1.26×10^{-4} M. The effect of K^+ on the protein was slightly antagonistic to that of Ca^{2+} , as indicated by u.v. difference spectroscopy and fluorescence titration.

INTRODUCTION

The highly acidic water-soluble S-100 protein (Moore, 1965) was originally considered to be a nervous-system-specific protein found primarily in the cytosol of glial cells (Ludwin *et al.*, 1976). However, S-100 has also been shown to be present in several non-neuronal tissues, such as melanocytes and Langerhans cells of skin (Cocchia *et al.*, 1981), chondrocytes (Stefansson *et al.*, 1982), stellate cells of the pituitary (Nakajima *et al.*, 1980), and adipose tissue (Marshak *et al.*, 1985).

S-100 protein in bovine brain is composed of three components: S-100a₀, S-100a and S-100b, with subunit composition $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ respectively. Both S-100a and S-100b exist in roughly equal proportions, whereas S-100a₀ represents only 3% of the total S-100 protein from this source (Isobe *et al.*, 1983). The amino acid sequence of S-100 protein indicates that there is extensive homology (58%) between the α - and β -subunits (Isobe & Okuyama, 1981). Furthermore, conservation of the primary structure is also extended to S-100 protein in other vertebrates, as recent findings show that human S-100b and rat S-100b differ from their bovine counterpart by only three and four amino acid residues respectively (Jensen *et al.*, 1985; Kuwano *et al.*, 1984). The α -subunit is also uniquely characterized by a tryptophan residue at position 90. Each of the subunits contains one Ca^{2+} -binding site at the C-terminus, similar to the EF model in parvalbumin (Tufty & Kretsinger, 1975). In addition, at pH 8.4 another Ca^{2+} -binding site becomes available, presumably at the N-terminus, resembling the I–II domains of bovine intestinal

Ca^{2+} -binding protein as described by Szebenyi *et al.* (1981).

The exact biological function of S-100 protein is not known (Moore, 1973). Publications from different laboratories suggest that S-100 proteins might be involved in regulating microtubule assembly and disassembly. However, S-100a and S-100b proteins have different effects on microtubule disassembly in the presence of low concentration of Zn^{2+} (Deinum *et al.*, 1983). For example, at low Zn^{2+} concentration S-100b induces disassembly and inhibits microtubule formation, whereas with S-100a no inhibition of assembly can be observed. Hence it is conceivable that S-100a and S-100b proteins have different physiological roles, and this could account for the existence of both isomers in the brain.

S-100 proteins undergo a conformational change in the presence of Ca^{2+} and Zn^{2+} (Mani *et al.*, 1982, 1983; Mani & Kay, 1983a, 1984; Baudier & Gérard, 1983; Umekawa *et al.*, 1983). Even though S-100a and S-100b have a similar affinities towards Ca^{2+} , subtle differences do exist in the microenvironment of specific chromophores. Similarly, upon binding Zn^{2+} a hydrophobic region is exposed in the case of S-100b, whereas S-100a is not affected. This principle has been utilized in developing an affinity column for purifying S-100a from S-100b (Baudier *et al.*, 1982).

The present study was initiated in the expectation that the observed differences in metal-ion-binding properties of S-100 proteins could be better understood by comparing the physicochemical properties of the α -subunit with those of the β -subunit. Accordingly, α -subunit was isolated from S-100a protein in the

* To whom correspondence and reprint requests should be addressed.

presence of 8 M-urea by anion-exchange chromatography. C.d., u.v. difference spectroscopy and fluorescence measurements were employed to explore the metal-ion (Ca^{2+} and Zn^{2+})-binding properties of the α -subunit. The spectral results obtained with the α -subunit have enabled us to compare its metal-ion-binding properties with those of S-100a ($\alpha\beta$) and S-100b ($\beta\beta$) proteins, and the comparison has revealed some interesting similarities as well as differences between the two subunits that may be related to their functional properties.

MATERIALS AND METHODS

Protein purification

The starting material (S-100a) was prepared from bovine brains by using the methodology described in an earlier paper (Mani *et al.*, 1982) for S-100b protein, with minor modifications. The solvent system used for the initial homogenization and subsequent operations involving the DEAE-cellulose column included 5 mM-2-mercaptoethanol. In order to improve the yield, the 40–95% -saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction, after heat treatment, was applied to a DEAE-cellulose column. The Sephacryl S-200 gel-filtration column was run in the presence of 0.1 M-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA, 0.4 M-NaCl, 6 M-urea and 5 mM-2-mercaptoethanol. Inclusion of 6 M-urea in the solvent was necessary, since in its absence a high- M_r protein (of M_r 50000) was co-eluted with S-100 proteins. S-100a protein was purified on a hydroxyapatite column.

Isolation of the α -subunit from S-100a protein

Purified S-100a protein was resolved into its components on a DEAE-Sephadex A-25 column (2.2 cm $\times$ 17 cm) in the presence of 50 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA, 0.1 M-NaCl, 8 M-urea and 15 mM-2-mercaptoethanol. A 60 mg portion of S-100a was applied to the column, and the α -subunit was eluted with a 0.1–0.31 M-NaCl gradient.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Weber & Osborn (1969). The final gel concentration was 15% acrylamide, 0.375% bisacrylamide. Electrophoresis was carried out in 6 mm $\times$ 100 mm tubes at 4 mA/tube for 11 h. Urea/SDS/polyacrylamide-gel electrophoresis followed the method of Swank & Munkres (1971), with the modification that the concentration of the urea was 6 M instead of 8 M. The final gel concentration was 12.5% polyacrylamide at a polyacrylamide/bisacrylamide ratio of 30:1. Electrophoresis was carried out at 3 mA/tube for 7 h. Standard polyacrylamide-gel electrophoresis was performed at pH 8.6 with Tris/glycine buffer as described by Schaub & Perry (1969).

Amino acid analysis

Samples that had undergone a 24 h hydrolysis in 6 M-HCl/0.1% phenol were analysed on a Durrum D-500 amino acid analyser (Dionex Corp.). Determination of half-cystine was by amino acid analysis of performic acid-oxidized protein (Glazer *et al.*, 1975). Tryptophan was determined spectrophotometrically by the method of Bencze & Schmid (1957).

Spectroscopy

U.v.-absorption and u.v. difference spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer over the wavelength range 350–250 nm with 1 cm-path-length cells. The spectra were measured at 25 °C with a Lauda thermoregulator connected to the sample compartment. Portions of perturbant, namely Ca^{2+} , K^{+} or Zn^{2+} , were added to the sample cell, and an equal volume of Chelex-100-treated water was added to the reference cell. The protein concentration was 0.2 mg/ml. Corrections were made for dilutions before the spectra were plotted.

C.d. measurements were made on a Jasco J500C instrument fitted with a DP-500N data processor as described previously (Mani & Kay, 1983a). A mean residue weight of 115 was used in calculating the molar ellipticity.

Fluorescence spectra were obtained with a Perkin-Elmer model MPF-44B spectrofluorimeter, and all measurements were made at 25 °C. The instrument was operated in the ratio mode with 6 nm band widths for excitation and emission slits. The A_{278} of the sample was less than 0.1. The protein was initially dissolved in 0.1 M-Tris/HCl buffer, pH 7.5, containing 4 mM-EDTA and 15 mM-2-mercaptoethanol, and thereafter was subjected to exhaustive dialysis with at least four changes against solvent that had been passed through a Chelex-100 column. Protein concentrations were determined from absorbance measurements, by using a molar absorption coefficient of $8300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm (Masure *et al.*, 1984).

RESULTS

Protein purification and characterization

S-100 proteins exist as heterodimers ($\alpha\beta$) or homodimers ($\beta\beta$) in native solvents, but under denaturing conditions they exist in their monomeric state (Mani *et al.*, 1982). For this reason, S-100a in 8 M-urea was applied on to a DEAE-Sephadex A-25 column. Under these conditions the α -subunit was eluted in the ascending portion of peak I (Fig. 1); peaks II and III also contained α -subunit, but with increasing amounts of β -subunit as a contaminant. The material in peak IV was

Fig. 1. Elution profile of the α -subunit of S-100a protein from a DEAE-Sephadex A-25 column

S-100a protein was applied to the column in 50 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA, 0.1 M-NaCl, 8 M-urea and 15 mM-2-mercaptoethanol. The α -subunit was eluted with a 900 ml linear gradient of 0.1–0.31 M-NaCl.

essentially β -subunit. The amount of protein recovered in peak I as α -chain corresponded to roughly 10% of the total S-100a protein applied to the column, and it was used exclusively for the studies described in the present paper. A comparison of the amino acid analysis of our α -chain preparation with that of Isobe & Okuyama (1981), as summarized in Table 1, revealed excellent agreement between the two preparations. The α -chain isolated from peak I was homogeneous according to polyacrylamide-gel electrophoresis in the presence as well as in the absence of SDS (Fig. 2). An M_r of 10000 ± 500 was deduced from its mobility in 15% SDS/polyacrylamide-gel electrophoresis (lane a), with reference to the mobilities of several protein standards. The standards were parvalbumin (M_r 11 200), calmodulin (M_r 16 500), cardiac troponin C (M_r 18 000), cardiac troponin I (M_r 27 000), cardiac troponin T (M_r 36 000) and creatine kinase (M_r 44 000). Urea/SDS/polyacrylamide-gel electrophoresis (lanes b and c) revealed the α -subunit in lane b to be free from any contamination by the β -subunit, which corresponded to the faster-migrating band in S-100a (lane c). Polyacrylamide-gel electrophoresis was also performed in the absence of SDS (lanes d and e). After alignment of the Bromophenol Blue dye front, it can be seen that the α -subunit moves faster in the presence of EDTA (lane d), as previously observed for S-100a and S-100b proteins (Mani *et al.*, 1982; Mani & Kay, 1983a). The decrease in mobility in the presence of Ca^{2+} (lane e) could be due to the neutralization of some of the negative charges resulting from cation binding. Ca^{2+} also induced the appearance of multiple bands, similar to those observed by Calissano *et al.* (1969). M_r measurements under non-denaturing conditions in the

Table 1. Amino acid composition of the α -subunit isolated from bovine brain S-100a protein based on a subunit M_r of 10400

Amino acid	Composition (residues/molecule of protein)		
	α -Subunit	α -Subunit*	β -Subunit*
Lys	8.7	9	8
His	2.0	2	5
Arg	0	0	1
Asp	12.6	13	9
Thr	4.0	4	3
Ser	5.3	5	5
Glu	15.6	15	19
Pro	0	0	0
Gly	6.6	6	4
Ala	8.0	8	5
Cys†	1.0	1	2
Val	7.2	8	7
Met	2.0	2	3
Ile	1.3	1	4
Leu	10.9	11	8
Tyr	2.1	2	1
Phe	4.8	5	7
Trp‡	1.2	1	0
		(93)	(91)

* From the amino acid sequence data of Isobe & Okuyama (1981).

† Determined by performic acid oxidation.

‡ Determined spectrophotometrically.

Fig. 2. Electrophoresis of either the α -subunit or S-100a protein of bovine brain under various conditions

Lane a, α -subunit in 15% SDS/polyacrylamide gel; lane b, α -subunit in 6 M-urea/SDS/polyacrylamide gel; lane c, S-100a in 6 M-urea/SDS/polyacrylamide gel; lanes d and e, α -subunit in 12.5% polyacrylamide gel in Tris/glycine buffer, pH 8.6, containing 1 mM-EDTA (d) and in the presence of 1 mM- CaCl_2 (e).

ultracentrifuge (Mani *et al.*, 1982) revealed slight aggregation in the presence of Ca^{2+} and Zn^{2+} ions. The weight-average M_r ranged from 12000 to 20000. However, in the absence of metal ions the protein existed almost entirely in the monomeric state.

U.v.-absorption spectrum

The u.v.-absorption spectrum of the α -subunit (Fig. 3) shows a peak at 278 nm with a shoulder at 283 nm, both of which are contributed by tyrosine chromophores. The appearance of a shoulder around 290 nm characterizes

Fig. 3. U.v.-absorption (—) and derivative spectra (----) of the α -subunit of S-100a protein in 0.1 M-Tris/HCl buffer, pH 7.5, at 25 °C

the presence of a tryptophan residue, which distinguishes the α -subunit from the β -subunit. This is also apparent in the derivative spectrum. However, the fine structures induced by phenylalanine residues around 260 nm are less prominent in the α -subunit compared with those in S-100a, since the phenylalanine/tyrosine ratio is only 5:2 for the α -chain as opposed to 12:3 for S-100a protein. The A_{278}/A_{280} ratio observed for the α -subunit is 1.65:1 compared with 1.5:1 for S-100a and 1.0:1 for S-100b.

C.d. studies

Representative far-u.v. c.d. spectra of the α -subunit in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol are shown in Fig. 4. The $[\theta]_{222}$ value of the apoprotein is -22100 ± 300 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$. Addition of Ca $^{2+}$ resulted in a 7.7% decrease to -20400 ± 300 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$. Subsequent addition of Zn $^{2+}$ to the same sample further decreased the $[\theta]_{222}$ value by another 1100 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$, which amounts to roughly 5%. Analysis of the c.d. data, by using the method of Chen *et al.* (1974), showed that the α -helical content decreased from 74% to 67% in the presence of Ca $^{2+}$, and this was further decreased by another 3% when Zn $^{2+}$ was added to the Ca $^{2+}$ -bound protein. On the other hand, addition of Zn $^{2+}$ to the apoprotein resulted in a decrease of 1400 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ or 6.25% in the $[\theta]_{222}$ value, and this was decreased by another 6% when Ca $^{2+}$ was subsequently added to the Zn $^{2+}$ -bound protein (see Table inset in Fig. 4). These results suggest that the order of metal ion addition to the α -subunit is not important, since in both cases the magnitude of change induced by one metal ion was not significantly influenced by the presence of the other, and the observed final c.d. spectra were essentially the same. In general, with S-100 proteins Ca $^{2+}$ addition results in a loss of α -helical content accompanied by unfolding of the molecule (Mani & Kay, 1983b). The Ca $^{2+}$ effect on the S-100 proteins is different when compared with other Ca $^{2+}$ -binding proteins such as troponin C, calmodulin and parvalbumin (Murray &

Fig. 4. Far-u.v. c.d. spectra of the α -subunit of S-100a protein in the absence of Ca $^{2+}$ in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol (—), in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol and 1 mM-CaCl $_2$ (---) and in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol, 0.97 mM-CaCl $_2$ and 0.388 mM-ZnSO $_4$ (.....)

The protein concentration was 0.19 mg/ml.

Fig. 5. Aromatic c.d. spectra of the α -subunit of S-100a protein in the absence of Ca $^{2+}$ in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol (—) and in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol and 1 mM-CaCl $_2$ (---)

The protein concentration was 0.88 mg/ml.

Kay, 1972; Walsh *et al.*, 1979). With the last-mentioned protein, an increase in apparent helical content is observed upon Ca $^{2+}$ addition.

The aromatic c.d. spectrum of the α -subunit is shown in Fig. 5. The negative band at 297 nm is due to the single tryptophan residue. The positive bands at 279 and 284 nm are attributed to tyrosine residues, and the two bands at 261.5 and 268.5 nm are assigned to the phenylalanine residues. From the spectrum it can be seen that the tryptophan residue at 297 nm and the tyrosine residues in the 280 nm region are perturbed upon Ca $^{2+}$ addition. Ellipticity values at 261.5 and 268.5 nm are also affected. On the other hand, addition of Zn $^{2+}$ to the α -subunit resulted in an alteration in the environment around the tyrosine residues only (results not shown).

U.v. difference spectroscopy

Absorption-difference spectroscopy was used to examine whether the environment of the aromatic residues is perturbed by Ca $^{2+}$ and Zn $^{2+}$ binding (Fig. 6). In the Ca $^{2+}$ -induced spectrum, the negative difference peak at 292.5 nm is characteristic of a blue-shift of the tryptophan absorption band. The double negative bands at 284.5 and 277.5 nm are typical of blue-shifts of tyrosine absorption bands. The blue-shifts associated with these residues are usually interpreted as arising from an increased exposure of these aromatic chromophores to solvent (Donovan, 1973). A similar finding is

Fig. 6. U.v. difference spectra of the α -subunit of S-100a protein in 0.1 M-Tris/HCl buffer, pH 7.5, containing 1.66 mM- CaCl_2 (—) and in 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.415 mM- ZnSO_4 (----)

The units are expressed as difference in molar absorption, $\Delta\epsilon$. The temperature was 25 °C. The protein concentration was 19.5 μM .

observed with calmodulin, which lacks tryptophan (Klee, 1977). In addition, a sharpening of the fine structures of the absorption bands below 270 nm is observed, suggesting that one or more of the phenylalanine residues are perturbed in the presence of Ca^{2+} . These findings are in agreement with our near-u.v. c.d. results mentioned above, whereby the environments of the tryptophan, tyrosine and phenylalanine residues are perturbed upon binding Ca^{2+} . Alternatively, these perturbations may also be due to neutralization of carboxy groups upon binding Ca^{2+} , and this accounts for the changes in the environment of the aromatic residues. When Zn^{2+} is subsequently added to the Ca^{2+} -bound protein, one of the two different peaks assigned to tyrosine residues became slightly more negative, suggesting that these residues move to a more polar environment (results not shown).

From the u.v. difference spectrum generated between the native subunit and Zn^{2+} -bound α -subunit, one can deduce that Zn^{2+} induces the exposure of tryptophan, tyrosine and phenylalanine residues to solvent, as evident from the blue-shifts of the absorption bands corresponding to these residues. However, the magnitudes of these blue-shifts are approx. 10% of those observed with Ca^{2+} , implying that the Zn^{2+} effect on the α -subunit is minimal compared with that of Ca^{2+} .

K^+ has a very small effect on the Ca^{2+} -induced u.v. difference spectrum, in agreement with the observations

made by Masure *et al.* (1984). The antagonistic effect is minimal, as reflected in the changes of the $\Delta\epsilon$ values upon K^+ addition (results not shown). For instance, in the absence of KCl, the $\Delta\epsilon$ values at 292 and 284 nm are $-574 \pm 11 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $-1024 \pm 11 \text{ M}^{-1} \cdot \text{cm}^{-1}$, and these decreased to $-473 \pm 11 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $-885 \pm 11 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at the corresponding wavelengths when KCl is subsequently added. In contradistinction, K^+ has a much larger antagonistic effect on S-100a and S-100b (Mani *et al.*, 1982; Mani & Kay, 1983a).

Fluorescence studies

When the α -subunit is excited at 280 nm, where both tyrosine and tryptophan residues absorb, its emission maximum is centred at $337 \pm 1 \text{ nm}$ (Fig. 7). The emission maximum at 337 nm is due to the partly buried tryptophan residue in the protein, and the shoulder around $307 \pm 1 \text{ nm}$ is due to the tyrosine residues. Addition of Ca^{2+} at pH 7.5 resulted in a 25% increase in the relative fluorescence intensity and the position of the emission maximum shifted to $340 \pm 1 \text{ nm}$ (i.e. a red-shift), suggesting that the tryptophan residue has become more exposed to solvent after the binding of Ca^{2+} , in agreement with our difference-spectral data. Baudier & Gérard (1983) have also reported a red-shift in the emission maximum of S-100a. By monitoring the percentage change in fluorescence intensity as a function of the amount of Ca^{2+} added (Fig. 8), a monophasic titration curve was obtained, indicating the existence of one class of Ca^{2+} -binding site at pH 7.5, with a K_d value of $1.26 \times 10^{-4} \text{ M}$. Ca^{2+} titration was also done in the presence of 90 mM-KCl; the titration curve obtained was identical in shape with that noted for Ca^{2+} , and a K_d value of $5.0 \times 10^{-4} \text{ M}$ was established. The slight decrease in the affinity for Ca^{2+} in the presence of KCl observed here, taken together with the difference-spectral data given above, suggested that K^+ only exhibits a small antagonistic effect towards Ca^{2+} on the α -subunit. The

Fig. 7. Fluorescence emission spectra of the α -subunit of S-100a protein in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol at 25 °C in the absence of Ca^{2+} (—) and in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol and 2.72 mM- CaCl_2 (----) at 25 °C

Fig. 8. Percentage change in fluorescence intensity at 337 nm as a function of the negative logarithm of Ca^{2+} concentration for the α -subunit of S-100a protein in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol (—) and in 0.1 M-Tris/HCl buffer, pH 7.5, containing 15 mM-2-mercaptoethanol and 90 mM-KCl (---) at 25 °C

The concentration of the protein was 5.9 μM .

affinity of the α -subunit for Ca^{2+} is similar to that reported for S-100a (Mani & Kay, 1983a). Calissano *et al.* (1974) have also reported that Ca^{2+} binding to an S-100 mixture resulted in an increase in fluorescence intensity. The K_d values obtained here are slightly lower than those reported by Masure *et al.* (1984): the latter investigators reported a K_d of 7×10^{-5} M in the absence of KCl and a K_d value of 3.4×10^{-4} M in the presence of 90 mM-KCl.

Owing to the small perturbation that Zn^{2+} had on the aromatic chromophores, we were unable to determine the affinity of the α -subunit for this metal ion by using the spectroscopic techniques described in the present paper.

DISCUSSION

In this paper we have described the isolation of the α -subunit from bovine brain S-100a protein. The methodology used is a modification of the procedure reported by Masure *et al.* (1984). Incorporation of 8 M-urea in the solvent system has enabled us to obtain a much improved resolution of the two subunits in the S-100a protein on a DEAE-Sephadex A-25 column. The α -subunit so isolated is homogeneous by the criteria of polyacrylamide-gel electrophoresis in the presence and in the absence of surfactants. The conformational changes induced upon binding Ca^{2+} and Zn^{2+} have been investigated. These results have enabled us to compare the metal-ion-binding properties of the α -subunit with

those of the β -subunit that exist in the S-100a heterodimer and in the S-100b homodimer.

In general, S-100 proteins undergo a conformational change upon binding Ca^{2+} and Zn^{2+} , but subtle differences exist in the manner in which the aromatic chromophores are being perturbed. For instance, with S-100a, the tryptophan and one or more tyrosine and phenylalanine residues become more exposed to solvent, whereas in S-100b the tyrosine and phenylalanine residues are buried (Mani & Kay, 1983b; Baudier & Gérard, 1983). Zn^{2+} binding also resulted in a gross conformational change in S-100a, whereas S-100b is not affected (Mani & Kay, 1984). The changes induced in the environment of the aromatic chromophores by Ca^{2+} and Zn^{2+} are the same in S-100b but different in S-100a. Ca^{2+} binding to S-100 proteins is pH-dependent, since at pH 8.3 an additional Ca^{2+} -binding site with a K_d value of 10^{-5} M becomes available. On the contrary, Zn^{2+} binding is independent of the pH (Mani & Kay, 1983b; Baudier & Gérard, 1983). K^{+} acts as an antagonist to Ca^{2+} binding (Calissano *et al.*, 1974; Mani & Kay, 1983b), but has no significant effect upon Zn^{2+} binding (Baudier & Gérard, 1983).

Ca^{2+} induced a conformational change in the α -subunit in a manner similar to that observed with S-100a and S-100b, namely a decrease in the α -helical content. Moreover, from fluorescence titration measurements the affinity of the α -subunit for Ca^{2+} is comparable with the values reported for S-100a and S-100b (Mani & Kay, 1983b). The Ca^{2+} effect on the microenvironment of the aromatic residues in the α -subunit is more akin to that in S-100a, but subtle differences do exist in the extent of these perturbations. In both proteins Ca^{2+} induced the exposure of the tryptophan, one or more tyrosine and phenylalanine residues; however, a comparison of their respective $\Delta\epsilon$ values in the u.v. difference spectra reveals that these residues are in a more-polar environment in the α -subunit than in S-100a. For instance, the $\Delta\epsilon$ value at 284 nm for the Ca^{2+} -induced spectrum of the α -subunit is $-1089 \text{ M}^{-1} \cdot \text{cm}^{-1}$ compared with $-650 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for S-100a (Mani & Kay, 1983a). This suggests that the Ca^{2+} effect on the aromatic chromophores in the α -subunit in S-100a is modified by the presence of the β -chain, in which the tyrosine and phenylalanine residues become buried, resulting in a lower observed $\Delta\epsilon$ value at the corresponding wavelengths for S-100a compared with the α -subunit.

In the presence of Zn^{2+} the α -subunit undergoes a conformational change. The fact that Zn^{2+} has no effect on S-100b in the far-u.v. c.d. region (Mani & Kay, 1984) suggests that the decrease in the α -helical content observed in S-100a upon binding this metal ion is localized in the α -subunit, in agreement with our far-u.v. c.d. results. The microenvironment of the aromatic residues in the α -subunit is also perturbed by Zn^{2+} binding. Near-u.v. c.d. studies suggest that only the tyrosine chromophores are affected to a small extent, in contrast with the large perturbations in both tyrosine and phenylalanine residues observed in S-100a (Mani & Kay, 1984). U.v. difference spectroscopy also shows that Zn^{2+} induces the exposure of the tryptophan, tyrosine and phenylalanine residues to a lesser extent in comparison with these residues in S-100a (Baudier & Gérard, 1983). The small conformational change observed with the α -chain could be due to a decrease in the number of histidine residues that can co-ordinate Zn^{2+} (Holmes &

Matthews 1981), i.e. two in the case of the α -subunit as opposed to seven in the S-100a protein.

Far-u.v. c.d. studies have demonstrated that the α -subunit can bind both Ca^{2+} and Zn^{2+} and that each metal ion is capable of inducing a conformational change in the presence of the other. In addition, the final decreases in $[\theta]_{222}$ values are similar irrespective of the order of addition of the metal ions. Furthermore, Zn^{2+} and Ca^{2+} induce different environments around the tyrosine residues, as depicted by near-u.v. c.d. These results may be taken as evidence to indicate that the two metal-ion-binding sites are different. Baudier & Gérard (1983) have also come to a similar conclusion with S-100a and S-100b, on the basis of their fluorescence studies.

On the basis of sequence homology with other Ca^{2+} -binding proteins, the Ca^{2+} -binding site on the α -subunit has been predicted to be in the region between residues 62 and 73 (Isobe & Okuyama, 1981). With the α -subunit, u.v. difference and fluorescence titration have shown that the tryptophan residue becomes exposed upon binding Ca^{2+} . Moreover, titration of the α -subunit with 5,5'-dithiobis-(2-nitrobenzoic acid), in the presence of Ca^{2+} , demonstrated that one thiol group/molecule of protein is labelled compared with none if EDTA is present (results not shown). Since the single tryptophan and cysteine residues occur at the C-terminal end of the α -chain at positions 90 and 85 respectively, this implies that the Ca^{2+} -binding site must be near these residues on the α -subunit.

In contradistinction, Zn^{2+} binding results in exposing a hydrophobic region at the N-terminal region only in the case of S-100b, and S-100a was not significantly affected by the presence of Zn^{2+} (Baudier & Gérard, 1983). The α -subunit in S-100a contains two tyrosine residues, at positions 26 and 74. Tyrosine-26 is in close proximity to the histidine residues, which are known to act as ligands for co-ordinating Zn^{2+} (Holmes & Matthews, 1981). Since we have observed that one or more tyrosine residue(s) is affected by Zn^{2+} , it is likely that tyrosine-26 is the one that is involved in Zn^{2+} binding on the α -subunit. Moreover, according to the near-u.v. c.d. and u.v. difference spectra, no significant perturbation of the tryptophan at the C-terminus was observed, suggesting that Zn^{2+} binding did not affect the C-terminus, where tyrosine-74 is located.

Fluorescence titration experiments in the presence of KCl indicated that the affinity of the α -subunit for Ca^{2+} is lowered slightly, as reflected in the observed K_d value, suggesting a small antagonistic effect similar to those observed in S-100a and S-100b (Baudier & Gérard, 1983; Mani *et al.*, 1982). However, the K^+ -induced u.v. difference spectrum suggested that this univalent cation had very little effect in perturbing the aromatic chromophores of the α -subunit, implying that the K^+ -binding site(s) is not in close proximity to these aromatic residues.

Far-u.v. c.d. studies of the α -subunit were also performed in the absence of any reducing agent (results not shown). We have observed that the final decrease in $[\theta]_{222}$ values, irrespective of the order of addition of metal ion, is larger (19%) compared with the 12% observed when 15 mM-2-mercaptoethanol is present in the solvent. This suggests that the state of the thiol group is important in conformational studies, as indicated in earlier studies (Calissano *et al.*, 1969; Mani & Kay, 1985a,b).

In conclusion, both S-100a and the α -subunit undergo a conformational change upon binding Ca^{2+} , resulting in a decrease in α -helical content as well as exposing the tryptophan, one or more tyrosine and phenylalanine residues to solvent. These residues are in a more-polar environment in the α -subunit compared with those in S-100a. This is probably due to the fact that the α -subunit is no longer under the influence of the β -chain, in which the aromatic chromophores become buried in the presence of Ca^{2+} . Although Zn^{2+} induces a conformational change upon binding to the α -subunit and S-100a, its effect on the aromatic chromophores in the α -subunit is minimal, with only the tyrosine residue affected to a small extent. The binding sites for the two metal ions appear to be different in the α -subunit. Despite the fact that the α -subunit shares an extensive sequence homology with the β -chain, these two subunits have different metal-ion-binding properties, which may be useful in probing structure-function relationships with these proteins in future studies.

We thank K. Oikawa and V. Ledsham for their excellent technical assistance and to M. Nattriss for performing the amino acid analyses. Vicki Luxton and Dawn Oare are thanked for typing the manuscript. I. K. M. L. thanks the Alberta Heritage Foundation for Medical Research for financial assistance in the form of a studentship.

REFERENCES

- Baudier, J. & Gérard, D. (1983) *Biochemistry* **22**, 3360–3369
- Baudier, J., Holtzcherer, C. & Gérard, D. (1982) *FEBS Lett.* **148**, 231–234
- Bencze, W. L. & Schmid, K. (1957) *Anal. Chem.* **29**, 1193–1196
- Calissano, P., Moore, B. W. & Friesen, A. (1969) *Biochemistry* **8**, 2318–2326
- Calissano, P., Alema, S. & Fasella, P. (1974) *Biochemistry* **13**, 4553–4560
- Chen, Y., Yang, J. T. & Chan, K. H. (1974) *Biochemistry* **13**, 3350–3359
- Cocchia, D., Michetti, F. & Donato, R. (1981) *Nature (London)* **294**, 85–87
- Deinum, J., Baudier, J., Briving, C., Rosengren, L., Wallin, M., Gérard, D. & Haglid, K. (1983) *FEBS Lett.* **163**, 287–291
- Donovan, J. W. (1973) *Methods Enzymol.* **27D**, 497–525
- Glazer, A. N., Delange, R. J. & Sigman, D. S. (1975) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), vol. 4, part 1, p. 23, North-Holland, Amsterdam
- Holmes, M. A. & Matthews, B. W. (1981) *Biochemistry* **20**, 6912–6920
- Isobe, T. & Okuyama, T. (1981) *Eur. J. Biochem.* **116**, 79–86
- Isobe, T., Ishioka, N., Masuda, T., Takahashi, Y., Ganno, S. & Okuyama, T. (1983) *Biochem. Int.* **6**, 419–426
- Jensen, R., Marshak, D. R., Anderson, C., Lukas, T. J., & Watterson, D. M. (1985) *J. Neurochem.* **45**, 700–705
- Klee, C. B. (1977) *Biochemistry* **16**, 1017–1024
- Kuwano, R., Usui, H., Maeda, T., Fukui, T., Yamanari, N., Ohtsuka, E., Ikehara, M. & Takahashi, Y. (1984) *Nucleic Acids Res.* **12**, 7455–7465
- Ludwin, S. K., Kosek, J. C. & Eng, L. F. (1976) *J. Comp. Neurol.* **165**, 197–208
- Mani, R. S. & Kay, C. M. (1983a) *Biochemistry* **22**, 3902–3907
- Mani, R. S. & Kay, C. M. (1983b) in *Calcium-Binding Proteins* (de Bernard, B., Sottocasa, G. L., Sandri, G., Carafoli, E., Taylor, A. N., Vanaman, T. C. & Williams, R. J. P., eds.), pp. 59–60, Elsevier, Amsterdam
- Mani, R. S. & Kay, C. M. (1984) *FEBS Lett.* **163**, 282–286
- Mani, R. S. & Kay, C. M. (1985a) *FEBS Lett.* **181**, 275–280

- Mani, R. S. & Kay, C. M. (1985b) *Biochem. Int.* **10**, 471–478
- Mani, R. S., Boyes, B. E. & Kay, C. M. (1982) *Biochemistry* **21**, 2607–2612
- Mani, R. S., Shelling, J. G., Sykes, B. D. & Kay, C. M. (1983) *Biochemistry* **22**, 1734–1740
- Marshak, D. R., Umekawa, H., Watterson, D. M. & Hidaka, H. (1985) *Arch. Biochem. Biophys.* **240**, 777–780
- Masure, H. R., Head, J. F. & Tice, H. M. (1984) *Biochem. J.* **218**, 691–696
- Moore, B. W. (1965) *Biochem. Biophys. Res. Commun.* **19**, 739–744
- Moore, B. W. (1973) in *Proteins of the Nervous System*, (Schneider, D. J., Angeletti, R. H., Bradshaw, R. A., Grasso, A. & Moore, B. W., eds.), pp. 1–26, Raven Press, New York
- Murray, A. C. & Kay, C. M. (1972) *Biochemistry* **11**, 2622–2627
- Nakajima, T., Yamaguchi, H. & Takahashi, K. (1980) *Brain Res.* **191**, 523–531
- Szebenyi, D. M. E., Obendorf, S. K. & Moffat, K. (1981) *Nature (London)* **294**, 327–332
- Schaub, M. C. & Perry, S. V. (1969) *Biochem. J.* **115**, 993–1004
- Stefansson, K., Wollmann, R. L., Moore, B. W. & Arnason, B. G. W. (1982) *Nature (London)* **295**, 63–64
- Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477
- Tufty, R. M. & Kretsinger, R. H. (1975) *Science* **187**, 167–169
- Umekawa, H., Endo, T. & Hidaka, H. (1983) *Arch. Biochem. Biophys.* **227**, 147–153
- Walsh, M., Stevens, F. C., Oikawa, K. & Kay, C. M. (1979) *Can. J. Biochem.* **57**, 267–278
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Received 27 January 1986/12 March 1986; accepted 7 April 1986