Studies on the α -subunit of bovine brain S-100 protein

H. Robert MASURE, James F. HEAD* and Harold M. TICE

Department of Physiology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, U.S.A.

(Received 12 July 1983/Accepted 2 November 1983)

A method is described for the rapid purification of both S-100 protein and calmodulin from crude bovine brain extracts by the use of a fluphenazine–Sephacryl column eluted stepwise with decreasing concentrations of free Ca^{2+} . Protein containing only α -subunit was purified from preparations of S-100 protein by anion-exchange chromatography. This protein co-migrated with the α -subunit of S-100 protein on sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis and had an amino acid composition identical with that previously reported for this subunit. The results of u.v.-absorption and fluorescence-emission spectroscopy indicate that the tryptophan residue of the purified α -subunit of S-100 protein undergoes a Ca^{2+} -induced change in environment. Measurements of changes in tryptophan fluorescence with increasing Ca^{2+} concentrations suggest an apparent dissociation constant of the α -subunit for Ca^{2+} of $7 \times 10^{-5} \text{ M}$ in the absence of K^+ . In the presence of 90 mM-K^+ this value is increased to $3.4 \times 10^{-4} \text{ M}$.

S-100 protein is a heterogeneous population of Ca^{2+} -binding proteins made up of two structurally related subunits, α and β . Sequence analysis of these subunits has shown that S-100 protein belongs to the family of homologous intracellular Ca^{2+} -binding proteins that includes calmodulin, troponin C and parvalbumins (Isobe *et al.*, 1978, 1981). Originally isolated from brain by Moore (1965), and thought to be specific to glial cells, S-100 protein has now been reported to be present in a number of tissues (Suzuki *et al.*, 1982; Hidaka *et al.*, 1983).

Various subunit compositions have been proposed for S-100 protein (see, e.g., Dannies & Levine, 1971; Mahadik *et al.*, 1979; Perumal & Murthi, 1981). The most widely accepted at this time is that described by Isobe *et al.* (1981), who have identified two isoforms: S-100a protein, consisting of one α -subunit and one β -subunit, and S-100b protein, consisting of two β -subunits.

The Ca^{2+} -dependent interactions of S-100 protein and calmodulin with matrix-bound drugs such as the phenothiazines and the naphthalene-sulphonamides have been exploited by a number of laboratories as a means of isolating these proteins (Marshak *et al.*, 1981; Endo *et al.*, 1981). In the present study we have employed fluphenazine–Sephacryl to bind calmodulin and S-100 protein

from crude brain extracts and have subsequently eluted each protein separately by stepwise elution with buffers containing decreasing concentrations of free Ca^{2+} .

Anion-exchange chromatography of the S-100 protein obtained from the phenothiazine–Sephacryl column gave three peaks of protein, containing respectively α -subunits alone, a mixture of α - and β -subunits, and β -subunits alone. In the present paper we describe the effects of Ca^{2+} and K^+ on the u.v.-absorption and fluorescence-emission spectra of the purified α -subunit.

Materials and methods

Materials

Fresh bovine brains were obtained from Trelogan's (Cambridge, MA, U.S.A.) and stored at -20°C until used. Fluphenazine was kindly given by Squibb and Sons (Princeton, NJ, U.S.A.). DEAE-Sephadex A-25 and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Chelex-100 was purchased from Bio-Rad Laboratories (Rockville Center, NY, U.S.A.). Dialysis tubing with an M_r cut-off of 6000 (Spectropor 2) was obtained from Fisher Scientific (Medford, MA, U.S.A.). EGTA was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

* To whom correspondence should be addressed.

Phenothiazine-Sephadex affinity chromatography

Fluphenazine was coupled to Sephadex 4B in accordance with the method of Charbonneau & Cormier (1979).

Buffers used for phenothiazine-Sephadex affinity chromatography contained 10 mM-Mops (4-morpholinepropanesulphonic acid)/KOH buffer, pH 7.2, 110 mM-KCl, 10 mM-NaCl and 15 mM-2-mercaptoethanol at 5°C. The column equilibration and wash buffer included an additional 1 mM-CaCl₂, whereas buffers used to elute Ca²⁺-dependent phenothiazine-binding proteins had different free Ca²⁺ concentrations adjusted by addition of calcium in 1 mM-EGTA. An apparent dissociation constant of 2.88×10^{-7} M for CaEGTA under these conditions was used in calculating free Ca²⁺ concentrations, this being based on the data of Fabiato & Fabiato (1979). The EGTA concentrations quoted in the text are nominal; free Ca²⁺ concentrations were calculated assuming the true EGTA concentrations to be 98% of nominal (Sigma).

Electrophoresis

Purity of proteins was assessed by sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis by the method of Swank & Munkres (1971). The 12.5% polyacrylamide (bisacrylamide/acrylamide ratio 1:15) slab gels (1 mm × 5 cm × 17.5 cm) were electrophoresed for approx. 9 h at 15 mA. Gels were stained in Coomassie Blue and destained in a 10% (v/v) methanol/10% (v/v) acetic acid solution.

Protein determination

The concentration of samples containing the purified α -subunit of S-100 protein was determined spectrophotometrically on the basis of a molar absorption coefficient of $8300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm. The concentration of other protein samples was determined by the method of Lowry *et al.* (1951), with a bovine serum albumin standard.

Spectroscopy

U.v.-absorption and difference spectra were recorded with a Beckman model 24 dual-beam spectrophotometer. Samples were analysed over a wavelength range of 240–300 nm, with 1 cm-light-path cells.

Fluorescence spectra were measured with a Perkin-Elmer model MPF 44E spectrofluorimeter. The amplifier was operated in the ratio mode with an excitation slit-width of 5 nm and an emission slit-width of 3 nm. Fluorescence-intensity measurements were as a routine standardized against a 10 μM solution of tryptophan in 5 mM-sodium phosphate buffer, pH 7.0. Ca²⁺ titrations of the changes in fluorescence were followed by

monitoring the peak tryptophan-residue emission signal at 345 nm while exciting at 290 nm.

Samples for spectral analysis were freed of Ca²⁺ by dialysis against 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-dithiothreitol and 1 mM-EGTA. The protein was then dialysed against Chelex-treated 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-dithiothreitol, either in the presence or in the absence of 90 mM-KCl, to remove EGTA.

Buffers used in Ca²⁺-titration studies were pre-treated by passage through a Chelex-100 column (2.5 cm × 10 cm) to remove contaminating Ca²⁺. Ca²⁺ was added to samples by micro-pipette as CaCl₂ solution; total additions did not exceed 2.5% of sample volume. After titration, sufficient EGTA was added to the samples to decrease free Ca²⁺ to approx. 0.1 μM . Spectra determined under these conditions were indistinguishable from those measured before Ca²⁺ additions, indicating that the titratable Ca²⁺-binding sites were unoccupied at the outset of the experiments.

Results

Protein preparations

Protein preparations were performed at 5°C unless otherwise noted. A 500 g portion of bovine brain was homogenized in a Waring blender with 500 ml of 10 mM-Mops buffer, pH 7.2, containing 110 mM-KCl, 10 mM-NaCl, 3 mM-EDTA and 15 mM-2-mercaptoethanol and centrifuged at 16000g for 90 min. The supernatant was decanted, adjusted to 10 mM-CaCl₂ and applied to a fluphenazine-Sephadex column (17 cm × 4.5 cm) equilibrated in 10 mM-Mops buffer, pH 7.2, containing 110 mM-KCl, 10 mM-NaCl, 1 mM-CaCl₂ and 15 mM-2-mercaptoethanol. Unbound material was washed from the column with 500 ml of the equilibration buffer.

Bound proteins were recovered from the column by stepwise elution with two buffers containing successively decreased free Ca²⁺ concentrations. The buffer steps contained the same components as the column-wash solution but included 1 mM-EGTA and different amounts of CaCl₂ to give free Ca²⁺ concentrations of 1 μM and 0.1 μM respectively (see the Materials and methods section).

Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis (Fig. 1) of protein eluted from the affinity column by the two low-Ca²⁺ steps revealed that the 1 μM -free-Ca²⁺ eluate contained two bands of protein having mobilities corresponding to those of the α -subunit and the β -subunit of S-100 protein. The 0.1 μM -free-Ca²⁺ eluate contained a single protein band with a mobility identical with that of calmodulin. This protein also activated bovine brain calmodulin-dependent

Fig. 1. Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis of samples from fluphenazine-Sepharose chromatography

Samples (150 μ l) of fractions of the eluate from the phenothiazine-Sepharose column were electrophoresed on 12.5%-polyacrylamide gels in the presence of sodium dodecyl sulphate and urea (see the Materials and methods section). Samples (a)–(d) are fractions eluted by buffer containing 1 μ M free Ca^{2+} . Samples (e)–(h) are fractions eluted by buffer containing 0.1 μ M free Ca^{2+} .

phosphodiesterase in a Ca^{2+} -dependent manner (results not shown).

The mixed S-100 protein components obtained from the phenothiazine-Sepharose column were further resolved by ion-exchange chromatography on DEAE-Sephadex. The S-100-protein-containing fractions from the affinity column were pooled and dialysed against three changes of 6 litres of 15 mM-2-mercaptoethanol. The dialysed pool was freeze-dried and reconstituted in 30 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 0.11 M-NaCl, 1 mM-EDTA and 15 mM-2-mercaptoethanol. This solution was applied to a DEAE-Sephadex A-25 column (2.5 cm $\times$ 10 cm) equilibrated in the same buffer. The column was washed with 200 ml of buffer, and a linear gradient of 0.11–0.34 M-NaCl in 800 ml of buffer was applied. Additional 0.34 M-NaCl in buffer was subsequently applied until the conductivity of the eluate was the same as that of the limit buffer. Measurement of the A_{280} of the eluted fractions showed three peaks of protein (Fig. 2). Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis of selected fractions from the eluate (Fig. 2) showed the first peak to

Fig. 2. Separation of S-100 proteins by anion-exchange chromatography

A 20.5 mg portion of S-100 protein, purified by phenothiazine-Sepharose affinity chromatography, was applied to a DEAE-Sephadex A-25 column and eluted with a linear salt gradient as described in the text. Three protein peaks were detected by measuring u.v. absorption at 280 nm (—), eluted between 0.22 M- and 0.28 M-NaCl (----). Each fraction contained 9 ml of eluate. Samples (200 μ l) of selected fractions from the eluate were analysed by sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis.

contain S-100 protein α -subunits whereas the second peak contained a mixture of S-100 protein α - and β -subunits. The declining slope of the third peak contained S-100 protein β -subunits.

The amino acid composition of protein from the first peak was found to be identical with that determined previously for S-100 protein α -subunit by sequence analysis (Isobe & Okuyama, 1981).

Quantitative determination

Determination of the amount of S-100 protein in the $1\text{ }\mu\text{M}$ -free- Ca^{2+} eluate from the fluphenazine-Sephadex column showed that 4.7 mg of S-100 protein was purified per 100 g of brain. About 2% of this amount was subsequently recovered from the DEAE-Sephadex column as purified α -subunit. Since the α -subunit makes up approx. 20% of total bovine brain S-100 protein (Isobe *et al.*, 1983), the yield of purified α -subunit represents about 10% of the total present; the remainder is eluted in combination with S-100 protein β -subunit. It was found that 9 mg of calmodulin per 100 g of brain was eluted from the phenothiazine-Sephadex affinity column in the $0.1\text{ }\mu\text{M}$ -free- Ca^{2+} eluate.

U.v.-absorption spectroscopy

Figs. 3(a) and 3(b) show the u.v.-absorption spectra of the purified α -subunit of S-100 protein and of the purified β -subunit (i.e. S-100b protein) prepared by the procedure described. The spectrum of the α -subunit shows a peak at 277 nm with a shoulder at 283 nm indicative of a contribution by tyrosine residues. The shoulder of tryptophan-residue absorption at 290 nm is scarcely detectable under these conditions. This spectrum is similar to that of S-100a protein (Isobe *et al.*, 1977; Baudier *et al.*, 1983). However, there is only a slight indication of phenylalanine-residue-induced secondary peaks from 268 nm to 250 nm, which are more prominent in the S-100a-protein spectrum. This is consistent with the relative content of aromatic amino acids in the α -subunit. The tryptophan/tyrosine/phenylalanine residue proportions for the purified α -subunit are 1:2:5, compared with 1:3:12 for S-100a protein (Isobe *et al.*, 1981). The absorption spectrum of S-100b protein displays a tyrosine-residue peak and shoulder at 277 nm and 283 nm respectively, and phenylalanine-residue fine structure (Isobe *et al.*, 1977; Mani *et al.*, 1982).

The difference spectrum of the purified α -subunit in the presence and in the absence of 2 mM- CaCl_2 is shown in Fig. 4. The minimum at approx. 294 nm corresponds to a blue-shift in the tryptophan-residue absorption, suggesting the exposure of this residue to a more-polar environment, presumably the solvent. The minima at 278 and 286 nm are due to blue-shifts in the absorption of tyrosine residues, also implying a net exposure of

Fig. 3. U.v.-absorption spectra of purified α -subunit of S-100 protein and purified β -subunit (i.e. S-100b protein). Spectra were measured with Ca^{2+} -free protein samples in 50 mM-Tris/HCl buffer, pH 7.6, containing 15 mM-2-mercaptoethanol. (a) Purified α -subunit at a concentration of $20\text{ }\mu\text{M}$ (on the basis of an M_r of 10400); (b) purified β -subunit (S-100b protein) at a concentration of $60\text{ }\mu\text{M}$ (on the basis of an M_r of 10500).

Fig. 4. U.v. difference spectrum of the purified α -subunit of S-100 protein in the presence and in the absence of Ca^{2+} . The purified α -subunit of S-100 protein was freed of Ca^{2+} as described in the Materials and methods section. Equal volumes of protein ($20\text{ }\mu\text{M}$) in 50 mM-Tris/HCl buffer, pH 7.6, containing 15 mM-2-mercaptoethanol were loaded into the reference cell and the sample cell. The contents of the sample cell was adjusted to 2 mM- CaCl_2 , and an equal volume of Chelex-treated water was added to the reference cell.

these residues in the presence of Ca^{2+} . The peaks at wavelengths below 275 nm suggest a perturbation in the environment of phenylalanine residues.

The addition of 90mM-KCl to the samples had no apparent effect on the Ca^{2+} -induced difference spectrum under these conditions.

Fluorescence spectroscopy

The fluorescence-emission spectrum of the purified α -subunit was typical of a tryptophan-containing protein, having an emission maximum at 345nm on excitation at 290nm.

Calissano *et al.* (1974) showed that Ca^{2+} binding to a mixture of S-100 proteins was paralleled by an increase in the intensity of fluorescence emission. Fig. 5 shows the relative change in fluorescence emission of the purified α -subunit at 345nm on addition of increasing amounts of Ca^{2+} . On taking the Ca^{2+} concentration required to produce a half-maximal change in fluorescence as an estimate of the dissociation constant, a value of $7 \times 10^{-5}\text{M}$ was obtained for the α -subunit in the absence of K^{+} . In the presence of 90mM-KCl this value was increased to $3.4 \times 10^{-4}\text{M}$.

Various values have been reported for the Ca^{2+} -binding affinities of the S-100 protein isoforms. The affinities are apparently dependent on the

concentrations of both H^{+} and K^{+} . The value determined in the present study for the purified α -subunit of S-100 protein in the absence of K^{+} at pH7.6 is identical with that determined by Calissano *et al.* (1974), using equilibrium dialysis, for mixed S-100 protein isoforms under similar conditions. However, our values are lower than those reported by Mani *et al.* (1982) for S-100b protein in the presence ($K_d = 8 \times 10^{-4}\text{M}$) and in the absence ($K_d = 2 \times 10^{-4}\text{M}$) of K^{+} .

Discussion

We describe a simple and rapid method for purifying calmodulin and the mixed isoforms of S-100 protein from crude extracts of bovine brain by the use of phenothiazine-Sepharose affinity chromatography. The separation of S-100 protein and calmodulin described here depends on the different affinities these proteins have for Ca^{2+} . S-100 protein was eluted at a free Ca^{2+} concentration of $1\text{ }\mu\text{M}$, and calmodulin was eluted at a concentration of $0.1\text{ }\mu\text{M}$. These values may give some indication of the relative affinities of the proteins for Ca^{2+} . However, the affinity of the immobilized protein-drug complex may differ from that of the protein in free solution. It is also possible that EGTA included in the buffers, to control free Ca^{2+} concentrations, may interact directly with the proteins (Haiech *et al.*, 1979; Kronman & Bratcher, 1983) and may therefore also influence their apparent affinities.

If the amounts of brain extract applied to the affinity column are increased significantly then the recovery of S-100 protein decreases. We attribute this to competition between calmodulin and S-100 protein for binding sites. Since calmodulin has a higher affinity for the phenothiazines than has S-100 protein (Marshak *et al.*, 1981), it will displace S-100 protein as saturation is approached.

Separation of protein containing only α -subunits was achieved by anion-exchange chromatography of preparations of mixed S-100 protein isoforms. Isobe *et al.* (1977) have previously used a prolonged isocratic elution of S-100 protein from DEAE-Sepharose in the presence of 5% (v/v) ethanol to separate S-100a protein from S-100b protein. They then separated the α - and β -subunits from S-100a protein by gel-filtration chromatography after aminoethylation of the proteins (Isobe *et al.*, 1981). In more-recent studies Isobe *et al.* (1983) have used high-pressure liquid-chromatographic procedures to characterize further the components present in the isocratic eluate from the DEAE-Sepharose column. Using this procedure, they have identified an additional isoform (S-100₀) containing only the α -subunit. This represents 3% of total S-100 protein, and is suggested by these workers to be an α -

Fig. 5. Ca^{2+} -induced change in the relative tryptophan-residue fluorescence of the purified α -subunit of S-100 protein

The emission maximum for the tryptophan-residue fluorescence of the purified α -subunit at 345nm on excitation at 290nm was measured with samples containing increasing CaCl_2 concentrations. Samples were in 50mM-Tris/HCl buffer, pH7.6, containing 15mM-2-mercaptoethanol in the absence (●) or in the presence (○) of 90mM-KCl. ΔF equals $[(F_c - F_0)/F_0] \times 100$, where F_c equals fluorescence intensity at a given CaCl_2 concentration and F_0 equals fluorescence intensity in the absence of Ca^{2+} . The concentration of α -subunit was $7.0\text{ }\mu\text{M}$ for samples in the presence of KCl and $6.5\text{ }\mu\text{M}$ for those in its absence. At saturating concentrations of Ca^{2+} the fluorescence-emission intensity at 345nm (measured in arbitrary units) was increased by 44% and 26%, in the presence and in the absence of 90mM-KCl respectively, over that of the Ca^{2+} -free protein.

subunit- α -subunit dimer. It seems likely that this isoform is identical with the protein described in the present study.

The changes in fluorescence-emission and u.v.-absorption spectra of the α -subunit on binding Ca^{2+} are consistent with a Ca^{2+} -induced conformational change leading to an exposure of the tryptophan residue. This is in agreement with results obtained by others with mixed isoforms of S-100 protein (Calissano *et al.*, 1974) or with S-100a (Baudier *et al.*, 1983). If the purified α -subunit exists in free solution as a dimer, as suggested by Isobe *et al.* (1983), it seems likely that the conformational change occurring on binding Ca^{2+} leads to a movement of the tryptophan residue similar to that detected in the α -subunit- β -subunit heterodimer S-100a protein (Baudier *et al.*, 1983).

The apparent dissociation constants of the purified α -subunit for Ca^{2+} determined in the present study are within the range of values determined by other workers for other forms of S-100 protein (Calissano *et al.*, 1974; Mani *et al.*, 1982). However, to determine whether there are significant differences in the affinities of the various forms under physiological conditions will require a more detailed analysis of the Ca^{2+} -binding properties of these proteins.

We thank Dr. Judith Marquis for use of the spectrofluorimeter and Mr. George Crombie for performing amino acid analysis. This work was supported in part by Grant PCM 8013009 from the National Science Foundation and by a Graduate Student Research Award from Boston University Division of Medical and Dental Sciences to H. R. M.

References

- Baudier, J., Mandel, P. & Gerrard, D. (1983) *J. Neurochem.* **40**, 145–152
- Calissano, P., Alema, S. & Fasella, P. (1974) *Biochemistry* **13**, 4553–4560
- Charbonneau, H. & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1039–1047
- Dannies, P. S. & Levine, L. (1971) *J. Biol. Chem.* **246**, 6276–6283
- Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. & Hidaka, H. (1981) *J. Biol. Chem.* **256**, 12485–12489
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Haiech, J., Derancourt, J., Pechere, J. F. & Demaille, J. G. (1979) *Biochemistry* **18**, 2752–2758
- Hidaka, H., Endo, T., Kawamoto, S., Yamada, E., Umekawa, H., Tanabe, K. & Hara, K. (1983) *J. Biol. Chem.* **258**, 2705–2709
- Isobe, T. & Okuyama, T. (1981) *J. Neurochem.* **37**, 522–524
- Isobe, T., Nakajima, T. & Okuyama, T. (1977) *Biochim. Biophys. Acta* **494**, 222–232
- Isobe, T., Tsugita, A. & Okuyama, T. (1978) *J. Neurochem.* **30**, 921–923
- Isobe, T., Ishioka, N. & Okuyama, T. (1981) *Eur. J. Biochem.* **115**, 469–474
- Isobe, T., Ishioka, N., Masuda, T., Takahashi, Y., Ganno, S. & Okuyama, T. (1983) *Biochem. Int.* **6**, 419–426
- Kronman, M. J. & Bratcher, S. C. (1983) *J. Biol. Chem.* **258**, 5707–5709
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mahadik, S. P., Korenovsky, A. & Rapport, M. M. (1979) *J. Neurochem.* **33**, 751–762
- Mani, R. S., Boyes, B. E. & Kay, C. M. (1982) *Biochemistry* **21**, 2607–2612
- Marshak, D. R., Watterson, D. M. & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6793–6797
- Moore, B. W. (1965) *Biochem. Biophys. Res. Commun.* **19**, 739–744
- Perumal, A. S. & Murthi, V. K. (1981) *Int. J. Biochem.* **13**, 799–804
- Suzuki, F., Nakajima, T. & Kato, K. (1982) *J. Biochem. (Tokyo)* **92**, 835–838
- Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477