Biochem. J. (1985) 232, 569–575 (Printed in Great Britain)

Physicochemical properties of a novel M_r-21000 Ca^{2+}-binding protein of bovine brain

John R. McDONALD,* Michael P. WALSH,* William D. McCUBBIN,† Kimio OIKAWA† and Cyril M. KAY†‡

*Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 3N1, and †Medical Research Council Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

The physicochemical properties of a novel M_r-21000 Ca^{2+}-binding protein isolated from bovine brain were investigated. The protein exhibited a partial specific volume of 0.724 ml/g, a degree of hydration of 0.47 g of water/g of protein and a mean residue weight of 119. Sedimentation equilibrium analysis revealed $M_r = 22600$ in the absence of Ca^{2+}; Ca^{2+} binding appeared to induce dimerization of the molecule. Size-exclusion chromatography indicated a compacting of the molecule on binding of Ca^{2+}: the Stokes radius decreased from 2.75 nm in the absence of Ca^{2+} to 2.56 nm in its presence. Far-u.v. c.d. spectroscopy showed the apoprotein to be composed of 44% α-helix, 18% β-pleated sheet and 38% random coil. Addition of either KCl (0.1 M) plus Mg^{2+} (1 mM), or Ca^{2+} (2 mM), changed the conformation to 49% α-helix, 18% β-pleated sheet and 33% random coil. Near-u.v. c.d. and u.v. difference spectroscopy both indicated perturbations in the environments of all three types of aromatic amino acids on binding of Ca^{2+}. Ca^{2+} binding also resulted in a 30% enhancement in the tryptophan fluorescence emission intensity. Ca^{2+} titration of the far-u.v. c.d. and fluorescence enhancement provided K_D values of 9.91 μM and 4.68 μM respectively. Finally, the protein was shown to bind Zn^{2+} with $K_D = 1.44$ μM (no Mg^{2+}) and 1.82 μM (+Mg^{2+}). These observations strongly support the possibility that this novel Ca^{2+}-binding protein resembles calmodulin and related Ca^{2+}-binding proteins and undergoes a conformational change on binding of Ca^{2+} which reflects a physiological role in Ca^{2+}-mediated regulation of brain function.

INTRODUCTION

It is well established that a number of structurally related Ca^{2+}-binding proteins are the major intracellular targets of the Ca^{2+} second message produced in response to an extracellular stimulus, e.g. nervous or hormonal stimulation (Cheung, 1980; Kretsinger, 1980; Means *et al.*, 1982; Burgess *et al.*, 1983; Walsh & Hartshorne, 1983). These Ca^{2+}-binding proteins transduce this second message into numerous specific physiological responses, e.g. smooth-muscle contraction (Walsh & Hartshorne, 1982) and neurotransmitter release (Knight & Scrutton, 1984; Knight *et al.*, 1984). Binding of Ca^{2+} to such regulatory Ca^{2+}-binding proteins, which have K_D values in the micromolar range, generally induces a change in conformation of the Ca^{2+}-binding protein (Levine & Dalgarno, 1983); the new conformer is then capable of interaction with a target enzyme or non-enzyme protein, this protein–protein interaction affecting the properties of the target protein. For example, the binding of Ca^{2+} to calmodulin induces rather extensive conformational changes (Wolff *et al.*, 1977; Dedman *et al.*, 1977; Klee, 1977; Walsh *et al.*, 1977, 1978, 1979; Seamon, 1980), including exposure of a hydrophobic site which is believed to be a site of interaction with target proteins (LaPorte *et al.*, 1980; Tanaka & Hidaka, 1980). The Ca^{2+}-dependent interaction of calmodulin with smooth-muscle myosin light-chain kinase, for example, converts this enzyme from an inactive into an active state (Dabrowska *et al.*, 1978) and leads ultimately to contraction.

Structural studies of various Ca^{2+}-binding proteins have revealed the existence of a family of homologous Ca^{2+}-binding proteins which includes calmodulin, troponin C, parvalbumin, S-100 protein, the intestinal vitamin D-dependent Ca^{2+}-binding protein, and others (Kretsinger, 1980). We isolated a novel M_r-21000 Ca^{2+}-binding protein (21 kDa CaBP) from bovine brain, utilizing Ca^{2+}-dependent hydrophobic-interaction chromatography (Walsh *et al.*, 1984). This Ca^{2+}-binding protein was found to interact with phenyl-Sepharose in a Ca^{2+}-dependent manner, to undergo a Ca^{2+}-dependent mobility shift on SDS/polyacrylamide-gel electrophoresis, to bind Ca^{2+} (1 mol/mol) with high affinity (K_D approx. 1 μM), to exhibit an amino acid composition similar to, but distinct from, those of previously described Ca^{2+}-binding proteins, and to exhibit a u.v. absorption spectrum similar to those of other Ca^{2+}-binding proteins (Walsh *et al.*, 1984; McDonald & Walsh, 1985). As part of a study aimed at complete characterization of this novel brain Ca^{2+}-binding protein, we report here the results of physico-chemical characterization of this protein, including demonstration of a Ca^{2+}-induced conformational change by c.d., u.v. and fluorescence spectroscopic techniques.

Abbreviations used: 21 kDa CaBP, M_r-21000 Ca^{2+}-binding protein; DTT, dithiothreitol; SDS, sodium dodecyl sulphate.

‡ To whom reprint requests should be addressed.

MATERIALS AND METHODS

Protein purification

The 21 kDa CaBP was purified from bovine brain by the procedure of Walsh *et al.* (1984), involving Ca^{2+}-dependent hydrophobic-interaction chromatography of the 100000 *g* supernatant, heat treatment and hydroxyapatite-column chromatography, followed by passage through a column of DEAE-Sephacel to eliminate traces of additional Ca^{2+}-binding proteins of M_r 17000 and 18400 (McDonald & Walsh, 1985). The protein was homogenous on 0.1%-SDS/7.5–20%-polyacrylamide-gradient slab-gel electrophoresis.

Analytical solutions were prepared by dialysis of solutions of 21 kDa CaBP in deionized water against the buffer of choice. In initial studies, 50 mM-Mops/1 mM-EGTA/1 mM-DTT, pH 7.0, was employed, but later experiments used 0.1 M-Tris/HCl/1 mM-DTT, pH 8.0, or 25 mM-Mops/1 mM-DTT, pH 7.0, which had been treated with Chelex-100 resin (Bio-Rad) to remove contaminating Ca^{2+} ions. In these solutions without EGTA, the protein solution was handled in acid-washed and Milli-Q (Millipore) water-rinsed plasticware. Before any spectroscopic measurement, all protein samples were clarified by centrifugation (Beckman model L8-70M with the 50 rotor; 30000 rev./min for 15 min). Protein concentrations were determined on a Perkin–Elmer Lambda 5 or a Cary 118C spectrophotometer by using absorption coefficient ($A^{1\%}_{277}$) of 9.1 (McDonald & Walsh, 1985).

Sedimentation analyses

Ultracentrifugation studies were done at 20 °C in a Beckman Spinco model E ultracentrifuge equipped with a photoelectric scanner, multiplex accessory, and high-intensity light source. Low-speed sedimentation-equilibrium runs were performed as described by Chervenka (1969), allowing at least 30 h to attain equilibrium. The rotor speed chosen for these runs was either 18000 or 20000 rev./min, depending on the protein concentration used. The solvent systems were 100 mM-Tris/HCl/1 mM-EGTA/1 mM-DTT, pH 8.0 (absence of Ca^{2+}), and 50 mM-Mops/1 mM-DTT/0.5 mM-$CaCl_2$ (presence of Ca^{2+}). In the Ca^{2+}-containing solvent, because of the presence of somewhat higher-M_r material, the rotor speed was decreased to 16000 rev./min.

Gel-filtration studies

Size-exclusion-chromatography experiments were performed at room temperature on a Varian Vista 5500 h.p.l.c. system equipped with a MicroPak TSK 2000 SW (7.5 mm × 30 cm) column. Elution profiles were monitored at 220 nm (0.1 absorbancy unit full scale), allowing the ready detection of as little as 10 μg of protein. The flow rate was programmed at 1.0 ml/min, and peak elution times were reported automatically. These elution times were converted into volumes. The following protein standards (Sigma) and Stokes radii (R_S) were used: catalase, 5.2 nm; lactate dehydrogenase, 4.1 nm; bovine serum albumin, 3.5 nm; ovalbumin, 2.84 nm; β-lactoglobulin, 2.66 nm; α-chymotrypsinogen, 2.19 nm; cytochrome *c*, 1.72 nm (as listed by Byers & Kay, 1982). The void volume (V_0) and total included volume (V_T) of the column were measured with solutions of ferritin and NaN_3 respectively. The standard proteins and the 21 kDa CaBP were run individually, and elution times/volumes were reproducible to better than 1%. The partition coefficient (σ) was calculated from the elution volume (V_e) by the relationship:

$$\sigma = (V_e - V_0)/(V_T - V_0)$$

The Stokes radius of 21 kDa CaBP ($R_{S,gel}$) was then calculated from a standard curve of log R_S versus σ as documented by Siegel & Monty (1966).

Fluorescence

Fluorescence spectra were taken on a Perkin–Elmer MPF-44B spectrofluorimeter with a thermostatically controlled cell holder maintained at 20 °C. Both emission and excitation slit band widths were usually set at 5 mm. No correction was made for any changes in detector response as a function of wavelength. Solution concentrations were adjusted so that the absorbance at the excitation wavelength (normally 280 nm) was always less than 0.05, thus eliminating the need to make any corrections for the inner filter effect.

Circular dichroism

These measurements were made on a Jasco 500-C spectropolarimeter with a DP 500 N data processor. The cell was maintained at 25 °C by means of a Lauda K-2/R circulating water bath. Far-u.v. (250–206 nm) scans were performed in a cell with a path length of 0.05 cm. The aromatic region (320–250 nm) was monitored with a micro cell which required only 90 μl and had a path length of 1 cm. Eight scans were run, with a time constant of 16 s. The computer-averaged trace was employed in all calculations. The equations of Chen *et al.* (1974), relating the observed ellipticity to the content of α-helix, β-structure and random coil, were used to compute the relative amounts of these conformers present. It is recognized that this analysis neglects some finer structures of proteins such as turn regions or short α-helical or β-sheet segments.

Absorption-difference spectra

Ca^{2+}-induced absorption difference spectra for 21 kDa CaBP samples were measured on a Perkin–Elmer Lambda 5 spectrophotometer in 1 cm path-length cells at ambient temperature over the wavelength range 350–250 nm. The zero baseline was established with 750 μl of protein solution (0.3–0.5 mg/ml) in both the sample and reference cells. Portions of concentrated $CaCl_2$ were added to the sample cell, and an equivalent volume of Milli-Q water was added to the reference cell. The full-scale absorbance range of 0.05 was used. Scan speeds were either 7.5 or 15 nm/min. The slit band width was maintained at 2 nm.

RESULTS

The sedimentation equilibrium of 21 kDa CaBP was studied in the absence of Ca^{2+} in 100 mM-Tris/HCl/1 mM-EGTA/1 mM-DTT, pH 8.0, at a protein concentration of 0.3 mg/ml. The scanning optical system operating at 280 nm was employed to record the distribution of protein within the cell at equilibrium. Linear plots were obtained of the natural logarithm of the protein concentration versus the square of the radial distance as shown in Fig. 1, and the weight-average M_r ($\bar{M}_w$) of 21 kDa CaBP calculated from such plots was 22600, independent of protein concentration across the cell. When these experiments were repeated in the presence of

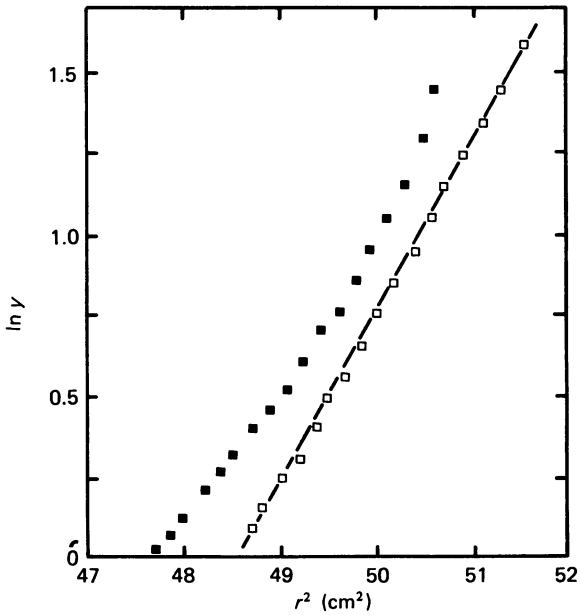

Fig. 1. Typical plots of ln y versus r^2 for 21 kDa CaBP

The initial loading concentration was 0.3 mg/ml, in 50 mM-Mops/1 mM-EGTA/1 mM-DTT, pH 7.0 (□), or in 50 mM-Mops/1 mM-$CaCl_2$/1 mM-DTT, pH 7.0 (■). The rotor speed for the run in the absence of Ca^{2+} was 20000 rev./min at 20 °C. $\overline{M}_w$, as determined from the least-squares slope of the line, was 22600. In the presence of Ca^{2+}, the rotor speed was decreased to 16000 rev./min at 20 °C.

Ca^{2+}, it was found that plots of ln y (where y is the concentration expressed in absorbance units) versus r^2 showed upward curvature across the cell. The $\overline{M}_w$ near the meniscus was estimated to be 23000, whereas near the cell bottom it had risen to about 40000.

Since the partial specific volume term ($\bar{v}$) is so important in M_r and hydrodynamic calculations, it would be desirable to have an accurate value for this parameter. A paucity of material precluded an experimental determination of $\bar{v}$ by densitometry; however, a value was calculated from the amino acid composition (McDonald & Walsh, 1985), as described by Cohn & Edsall (1943). The value obtained was 0.724 ml/g, and the degree of hydration of the protein particle was estimated to be 0.47 g of water/g of protein from the amino acid composition (Kuntz & Kauzmann, 1974).

The Stokes radius of 21 kDa CaBP was determined by size-exclusion chromatography, in the absence and presence of Ca^{2+}, by using h.p.l.c. equipment and methodology. Single runs were effected in 15 min. In both the apo and Ca^{2+}-loaded forms, 21 kDa CaBP was eluted from a TSK 2000 SW column in a single symmetrical peak. The apparent Stokes radius ($R_{S,gel}$) of 21 kDa CaBP in 100 mM-NaCl/25 mM-Mops/0.5 mM-EGTA/0.1 mM-DTT, pH 7.0, i.e. in the absence of Ca^{2+}, was 2.75 nm, whereas in the presence of Ca^{2+}, in a solvent system of 100 mM-NaCl/25 mM-Mops/1 mM-$CaCl_2$/0.5 mM-DTT, pH 7.0, it decreased to 2.56 nm.

The far-u.v. c.d. spectrum of 21 kDa CaBP in 25 mM-Mops/1 mM-DTT, pH 7.0, is shown in Fig. 2(*a*). The spectrum of the apoprotein shows the typical bimodal spectrum of an α-helix-containing protein. The

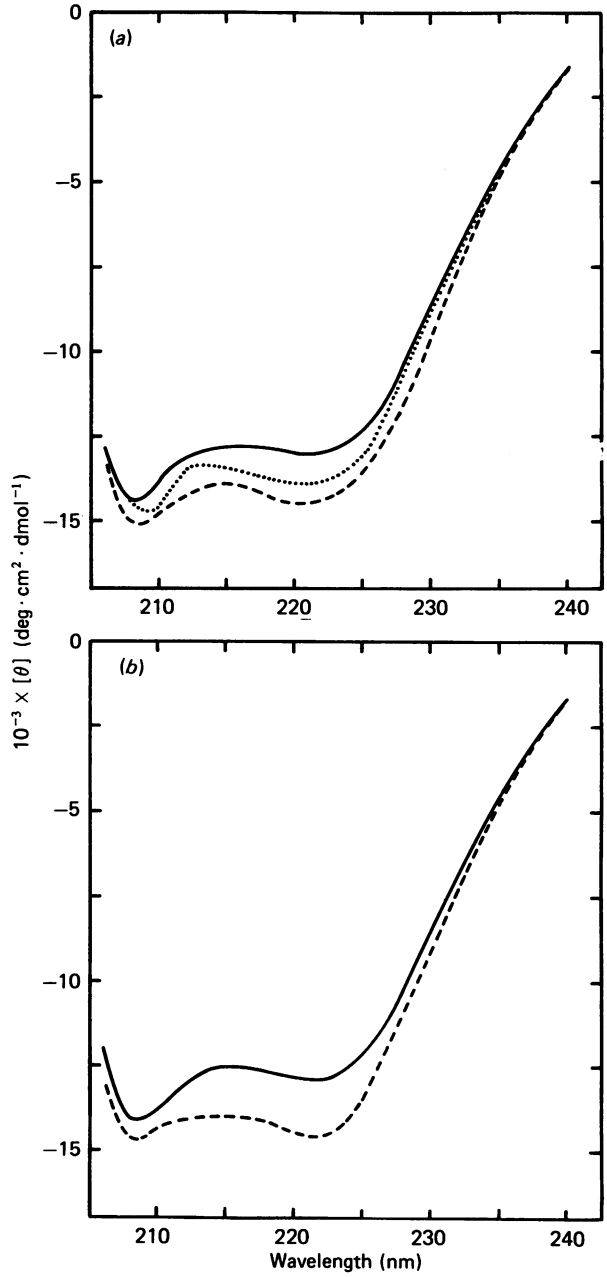

Fig. 2. Far-u.v. c.d. spectra of 21 kDa CaBP

(*a*) Spectra in 25 mM-Mops/1 mM-DTT, pH 7 (apo state) (——), in 25 mM-Mops/100 mM-KCl/1 mM-DTT, pH 7 (····), and in 25 mM-Mops/100 mM-KCl/2 mM-$CaCl_2$/1.0 mM-DTT, pH 7 (– – – –). The protein concentration was 0.35 mg/ml, the path length of the cell was 0.05 cm, and a mean residue weight of 119, as calculated from the amino acid composition, was employed in ellipticity calculations. (*b*). Spectra in 25 mM-Mops/1 mM-DTT, pH 7.0 (apo state) (——), and in 25 mM-Mops/100 mM-KCl/1 mM-$MgCl_2$/1 mM-DTT, without or with 2 mM-$CaCl_2$ (– – – –). The protein concentration was 0.34 mg/ml and the path length of the cell was 0.0503 cm.

two other spectra in Fig. 2(*a*) reveal the effect of adding KCl rendered Ca^{2+}-free by treatment with Chelex-100 (to 100 mM) and excess $CaCl_2$ to the protein. Fig. 2(*b*) illustrates the effect of adding KCl (100 mM) and $MgCl_2$ (1 mM) to the apoprotein. The subsequent addition of

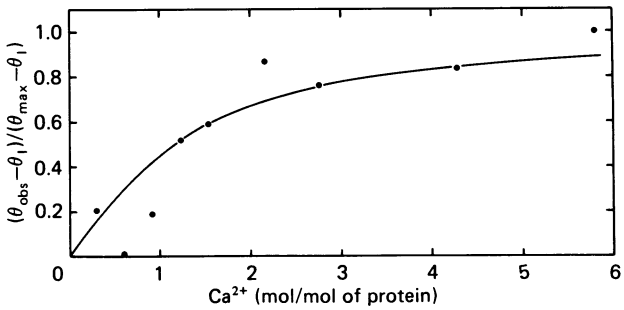

Fig. 3. Far-u.v. c.d. Ca^{2+} titration of 21 kDa CaBP

Solvent was 25 mM-Mops/1 mM-DTT, pH 7, which had been treated with Chelex-100 resin to remove residual Ca^{2+}. $[\phi]_{221}$ was monitored. The continuous curve was computed by assuming one binding site with $K_D = 9.91$ μM. Protein concentration was 0.34 mg/ml.

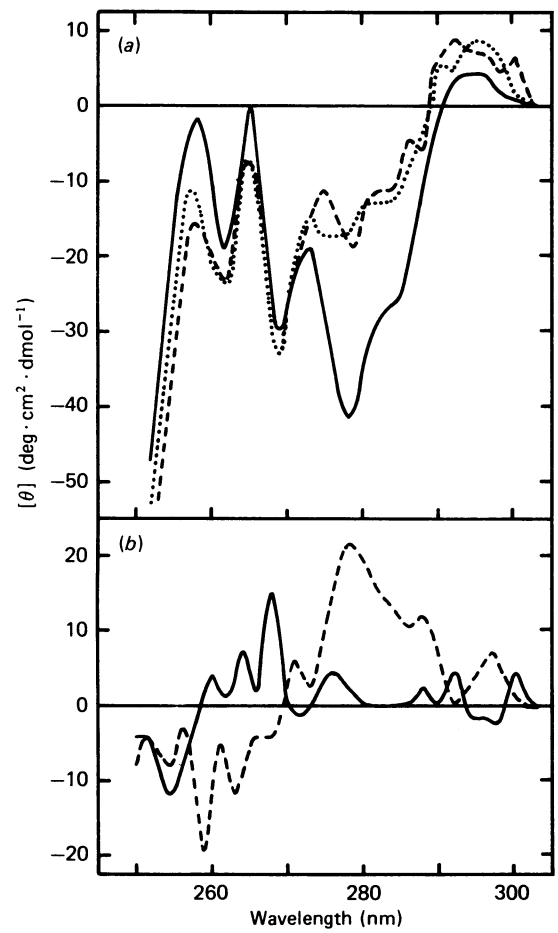

Fig. 4. Near-u.v. c.d. spectra of 21 kDa CaBP

(*a*) Spectra in Chelex-100-treated 25 mM-Mops/1 mM-DTT, pH 7 (apo state) (——), in 25 mM-Mops/2 mM-$CaCl_2$/1 mM-DTT, pH 7 (····), and in 25 mM-Mops/100 mM-KCl/2 mM-$CaCl_2$/1 mM-DTT, pH 7 (– – – –). Protein concentration was 0.35 mg/ml and a micro cell was used with a pathlength of 1 cm. (*b*) This shows the same data as in (*a*) plotted in the form of difference spectra: Ca^{2+}-loaded 21 kDa CaBP − apo form (– – – –); Ca^{2+}/KCl-loaded protein − Ca^{2+}-loaded protein (——).

$CaCl_2$ to this solution produces essentially no further spectral alterations. If these ellipticity values are translated into amounts of α-helix, it appears that apo-(21 kDa CaBP) has some $44 \pm 1\%$ of this conformer. Addition of K^+/Mg^{2+} or Ca^{2+} raises this to $49 \pm 1\%$.

The Ca^{2+}-induced change in $[\theta]_{222}$ was found to be some 1600°, which is large enough to allow a titration to be performed. The results of such a representative titration are shown in Fig. 3. The data were analysed by a computerized non-linear least-squares fit (continuous line) to yield a K_D value of $9.9 (\pm 0.9) \times 10^{-6}$ M (regression value ± S.E.M.). These measurements were performed with the apoprotein dissolved in 25 mM-Mops/1 mM-DTT, pH 7.0.

Fig. 4(*a*) shows the c.d. spectrum for 21 kDa CaBP in the 310–250 nm (aromatic) spectral region. The spectrum of the apoprotein revealed a small positive peak centred around 295 nm, which presumably arises from tryptophan. A fairly large negative trough near 280 nm probably represents contributions from tyrosine residues, and the peaks near 273, 265 and 258 nm along with the troughs near 269 and 263 nm may be assigned to phenylalanine residues in an asymmetric environment. Addition of saturating concentrations of Ca^{2+} produced an approximate doubling in intensity of the 295 nm peak, along with a dramatic decrease (to about one-third of the initial value) in the magnitude of the 280 nm trough. A sharpening effect and overall decrease was also noted in signal intensity of the bands between 250 and 270 nm. Addition of KCl to 100 mM to the Ca^{2+}-loaded protein

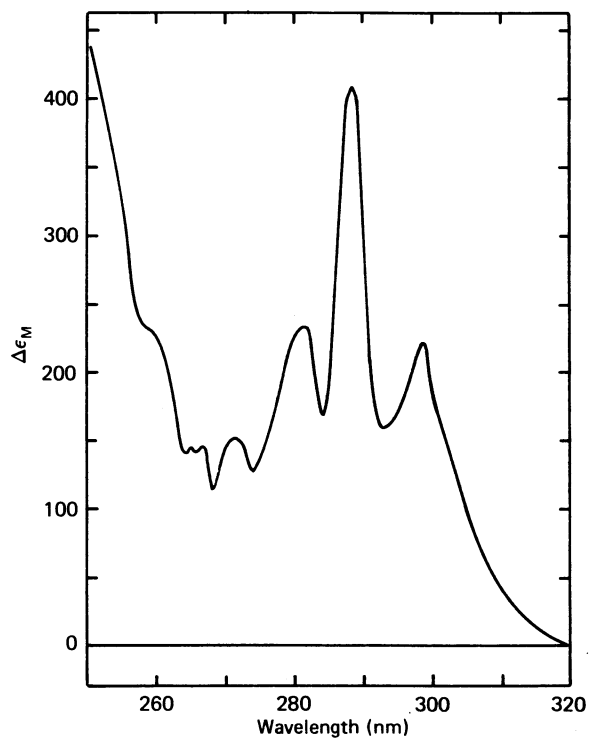

Fig. 5. U.v. difference absorption spectrum of 21 kDa CaBP produced by Ca^{2+}

The solvent was Chelex-100-treated 100 mM-Tris/HCl/1 mM-DTT, pH 7.0. Protein concentration was 0.301 mg/ml and $CaCl_2$ was added to a final concentration of 0.76 mM. Cell path length was 1 cm, and temperature 22 °C.

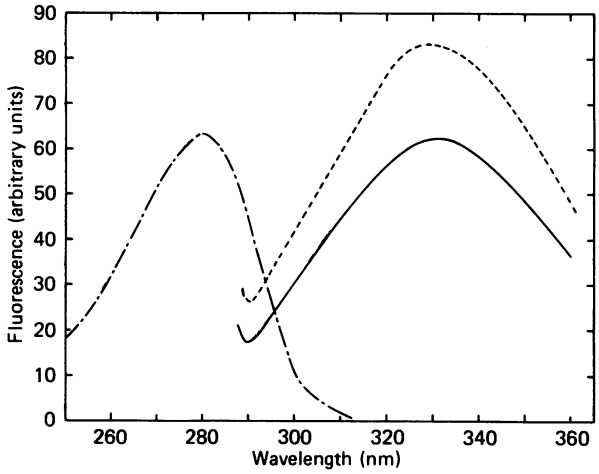

Fig. 6. Fluorescence excitation and emission spectra for 21 kDa CaBP

The excitation spectrum (-·-·-·) was measured in 50 mM-Mops/1 mM-EGTA/1 mM-DTT, pH 7.0. The emission spectrum of the apoprotein was measured in the same solvent (——). Addition of 1 mM-$MgCl_2$ produced no alteration to the spectrum. The spectrum of the Ca^{2+}-loaded protein (– – –) is also shown. Concentration of protein was 0.026 mg/ml.

seemed to produce a slight alteration in the bands corresponding to phenylalanine. These effects are illustrated more dramatically in the form of difference spectra as shown in Fig. 4(*b*).

Additional information regarding the binding of Ca^{2+} to the 21 kDa CaBP was gleaned from absorption difference-spectrum measurements carried out in 100 mM-Tris/HCl/1 mM-DTT, pH 8.0. A typical spectrum generated by the presence of a saturating amount of Ca^{2+} is displayed in Fig. 5. Positive peaks were noted near 298, 288, 281 and 272 nm, with shoulders in the vicinity of 265 and 258 nm. These suggest perturbations in the environments of tryptophan, tyrosine and phenylalanine residues.

Fluorescence excitation and emission spectra for 21 kDa CaBP are displayed in Fig. 6. The excitation spectrum showed a maximum at 280 nm. The emission maximum was centred near 330 nm, typical for a protein with buried tryptophan residues. The emission spectrum was unaffected in both the position and the intensity of the emission maximum, by the addition of $MgCl_2$ to 1 mM. The addition of saturating amounts of $CaCl_2$ produced some 30% increase in the emission intensity. This effect may be titrated in an analogous manner to the c.d. data. Measurements were initially done in 50 mM-Mops/1 mM-DTT/1 mM-EGTA, pH 7.0; the data were analysed and yielded $K_D = 1.4\ (\pm 0.1) \times 10^{-6}$ M. When the apo state was generated by dialysis against Chelex-treated 25 mM-Mops/1 mM-DTT, pH 7.0, curve fitting of the resulting fluorescence data yielded $K_D = 4.1\ (\pm 0.4) \times 10^{-6}$ M. Addition of $MgCl_2$ to 1 mM gave approximately the same value, $K_D = 5.4\ (\pm 0.5) \times 10^{-6}$ M. In the presence of 100 mM-KCl and 1 mM-$MgCl_2$, $K_D = 4.7\ (\pm 0.5) \times 10^{-6}$ M. The binding of Zn^{2+} was also followed by fluorescence titration. Note that it is essential to remove DTT from these solvents before addition of Zn^{2+}. Failure to do this caused immediate precipitation, with consequent invalidation of any collected data. To protect thiol groups in the protein under these conditions, β-mercaptoethanol was used. In 20 mM-Mops/28 mM-β-mercaptoethanol, pH 7.0, $K_D = 1.4\ (\pm 0.1) \times 10^{-6}$ M. Addition of 1 mM-$MgCl_2$ before Zn^{2+} titration produced $K_D = 1.8\ (\pm 0.2) \times 10^{-6}$ M.

DISCUSSION

The M_r of the 21 kDa CaBP as determined by sedimentation-equilibrium analysis was found to be 22600 in the absence of Ca^{2+}. This is in close agreement with the value obtained by SDS/polyacrylamide-gel electrophoresis (M_r 22000 in the absence of Ca^{2+} and M_r 21000 in the presence of Ca^{2+}; Walsh *et al.*, 1984). Furthermore, no evidence was found for dissociation or particle aggregation occurring under the conditions of these sedimentation equilibrium experiments. In the presence of excess Ca^{2+} (0.5 mM), it was found that the $\overline{M}_w$ increased across the cell from the monomer value of 23000 near the meniscus to approx. 40000 at the cell bottom. Thus Ca^{2+} seems to have induced self-association of the protein to the dimer state. This finding is reminiscent of previous observations on the Ca^{2+}-binding protein from skeletal muscle, troponin C, that suggested a combined Ca^{2+}-mediated and -facilitated dimerization of the protein (Margossian & Stafford, 1982).

The decrease in Stokes radius ($R_{S,gel}$) for 21 kDa CaBP from 2.75 nm in the metal-free state to 2.56 nm in the Ca^{2+}-loaded form implied a compacting of the molecule as the metal was bound. These values may be compared with 2.63 nm (apo state) and 2.43 nm (Ca^{2+}-loaded state) noted for bovine cardiac troponin C by Byers & Kay (1982), or the values of 2.14 nm and 2.09 nm quoted for calmodulin in its apo and Ca^{2+}-loaded states respectively by Klee *et al.* (1980).

The successful use of Ca^{2+}-dependent hydrophobic interaction chromatography in the isolation of the 21 kDa CaBP indicated that the secondary structure of the protein was influenced by this metal ion (Walsh *et al.*, 1984). The c.d. studies reported herein have confirmed this idea. The apoprotein has some 44% α-helix, 18% β-pleated sheet and 38% random coil, whereas the protein in the presence of excess Ca^{2+} has 49% α-helix, 18% β-structure and 33% random coil. It was noted that the structure of the protein was also sensitive to the presence of K^+ ions, since a comparable structural increase was noted on addition of 100 mM-KCl or of 100 mM-KCl/1 mM-$MgCl_2$.

This change in secondary structure can be compared with that induced by Ca^{2+} in the folding of the muscle Ca^{2+}-binding protein troponin C. This protein in the apo state has some 35% α-helix, whereas on addition of excess Ca^{2+} the α-helix content rose to about 50% (Murray & Kay, 1972; McCubbin & Kay, 1973). In this protein, increase in ionic strength, provided that no Ca^{2+} was inadvertently introduced, had little effect on the amount of secondary structure.

The metal-induced increase in α-helix found for 21 kDa CaBP can be compared with data from other Ca^{2+}-binding proteins isolated from brain tissue. The results of several far-u.v. c.d. studies of calmodulin, summarized by Klevit (1983), indicate that the α-helical content of calmodulin in the absence of Ca^{2+} is 30–45% and that it increases by 9–16% upon Ca^{2+} binding. Other experiments show that the secondary structure is unaffected by an increase in

ionic strength to 0.5 M (Walsh *et al.*, 1979). In contrast with these data, it was shown that the apparent α-helix content of brain S-100b protein was decreased from 58% to 52% in the presence of Ca^{2+}. Addition of 90 mM-KCl seemed to produce a slight recovery of the secondary structure (Mani *et al.*, 1982). Similarly, the S-100a protein underwent a decrease in apparent α-helical content, from 54% to 49%, on Ca^{2+} addition at pH 8.3. At pH 7.5, this effect, though similar, was decreased somewhat in intensity (Mani & Kay, 1983).

It is possible to extend these comparisons into the near-u.v. spectral region, i.e. the region from 250–320 nm dominated by the signals from asymmetrically situated aromatic amino acids. As was demonstrated by Walsh *et al.* (1979) and Crouch & Klee (1980), Ca^{2+} binding affects the environment of both tyrosyl and phenylalanyl residues in bovine brain calmodulin, as reflected by changes in the near-u.v. c.d. spectrum. For calmodulin from *Neurospora crassa*, which has only a single tyrosine residue, presumably located in position 138, the Ca^{2+}-saturated protein displayed a broad negative trough above 273 nm, whereas upon metal depletion the sign of the intensities of the tyrosine transitions was reversed, suggesting that in the metal-free form of this calmodulin the tyrosine conformation was stabilized in a dissymmetric environment of opposite type to that of the Ca^{2+} form (Cox *et al.*, 1982).

For brain S-100b protein, it was found that addition of Ca^{2+} perturbed the environment of both tyrosine and phenylalanine residues. Addition of K^+ was shown to have an antagonistic effect to that of Ca^{2+} (Mani *et al.*, 1982). When the S-100a isomer was studied, it was noted that addition of Ca^{2+} perturbed the tryptophan band at 295 nm, the tyrosine signal in the 280 nm region, as well as the signals corresponding to phenylalanine at 269 and 262 nm (Mani & Kay, 1983).

U.v. difference spectroscopy has confirmed these observations. For S-100b protein, peaks at 287 and 280 nm in the difference spectrum produced by Ca^{2+} addition were attributed to perturbation of the single tyrosine residue. Peaks between 250 and 270 nm indicated environmental differences in one or more of the phenylalanine chromophores as Ca^{2+} was added (Mani *et al.*, 1982). Klee (1977) noted similar observations with calmodulin. For S-100a protein, the interesting finding was noted that the bands corresponding to tryptophan and tyrosine were negative, indicating that the microenvironment around these chromophores was different in the two proteins (Mani & Kay, 1983).

Thus the findings noted in the present study, by both near-u.v. c.d. and difference spectroscopy, namely that Ca^{2+} induced perturbations in the environments of all three types of aromatic amino acid, are certainly in line with previous observations on similar systems. The fact that the entire u.v. difference spectrum for 21 kDa CaBP was positive or 'red-shifted' would imply burying of the chromophores in a more non-polar environment as the metal was added. Alternatively, the charge neutralization induced by Ca^{2+} might have perturbed the environment around these moieties in a similar fashion.

The fluorescence emission spectrum for 21 kDa CaBP is typical of a protein containing tryptophan. The emission maximum for the protein is centred near 330 nm, which is indicative of the tryptophan residues being incompletely exposed to the solvent. Although no change could be detected in the wavelength of the emission maximum on Ca^{2+} addition, there was an approx. 30% increase in fluorescence intensity. This increase may be equated with the increase in helix noted from c.d. measurements. It is noteworthy that Mg^{2+} produced no change in the fluorescence signal.

The Ca^{2+}-binding parameters of the 21 kDa CaBP had been determined by equilibrium dialysis using $^{45}CaCl_2$. In the presence of 3 mM-$MgCl_2$ and 0.15 M-KCl, the protein bound approx. 1 mol of Ca^{2+}/mol of protein, with an apparent K_D of approx. 1 μM (McDonald & Walsh, 1985). When the Ca^{2+}-induced change in either $[\phi]_{222}$ or F_{330} (fluorescence) was titrated, and the resulting data were handled by non-linear least-squares analysis, assuming that 1 mol of Ca^{2+} was bound per mol of protein, somewhat higher values were found for K_D. From the fluorescence data, $K_D = 4.68$ μM, whereas from c.d. observations, $K_D = 9.91$ μM. Initial fluorescence experiments, where the titration was done in 50 mM-Mops/1 mM-DTT/1 mM-EGTA, pH 7.0, gave $K_D = 1.41$ μM, but since there appeared to be additional binding at a site with $K_D \cong 2.5 \times 10^{-4}$ M, which seemed possibly to be an artefact, it was decided to generate the apo state with the use of Chelex-100 treatment of buffers etc. and thereby to eliminate EDTA/EGTA from the solutions.

In conclusion, these spectral and hydrodynamic observations support the possibility that this novel Ca^{2+}-binding protein resembles calmodulin, troponin C and S-100 in undergoing a Ca^{2+}-induced conformational change, and mimics S-100 in that its conformation is sensitive to Zn^{2+} binding as well. These changes may well reflect a physiological role for this protein in Ca^{2+}- and Zn^{2+}-mediated regulation of brain function.

This work was supported by grants (to C.M.K. and M.P.W.) from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research, J.R.M. is the recipient of a postdoctoral fellowship from the Alberta Heritage Foundation for Medical Research.

REFERENCES

Burgess, W. H., Schleicher, M., van Eldik, L. J. & Watterson, D. M. (1983) in Calcium and Cell Function (Cheung, W. Y., ed.), vol. 4, pp. 209–261, Academic Press, New York
Byers, D. M. & Kay, C. M. (1982) Biochemistry **21**, 229–233
Chen, Y. H., Yang, J. T. & Chou, K. H. (1974) Biochemistry **13**, 3350–3359
Chervenka, C. H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, Spinco Division of Beckman Instruments, Palo Alto, CA
Cheung, W. Y. (1980) Science **207**, 19–27
Cohn, E. J. & Edsall, J. T. (1943) Proteins, Amino Acids and Peptides, pp. 370–381, Reinhold, New York
Cox, J. A., Ferraz, C., Demaille, J. G., Ortega Perez, R., Van Tuinen, D. & Marmé, D. (1982) J. Biol. Chem. **257**, 10694–10700
Crouch, T. H. & Klee, C. B. (1980) Biochemistry **19**, 3692–3698
Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. & Hartshorne, D. J. (1978) Biochemistry **17**, 253–258
Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) J. Biol. Chem. **252**, 8415–8422
Klee, C. B. (1977) Biochemistry **16**, 1017–1024
Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) Annu. Rev. Biochem. **49**, 489–515
Klevit, R. E. (1983) Methods Enzymol **102**, 82–104
Knight, D. E. & Scrutton, M. C. (1984) Nature (London) **309**, 66–68

Knight, D. E., Niggli, V. & Scrutton, M. C. (1984) Eur. J. Biochem. **143**, 437–446
Kretsinger, R. H. (1980) CRC Crit. Rev. Biochem. **8**, 119–174
Kuntz, I. D. & Kauzmann, W. (1974) Adv. Protein Chem. **28**, 239–345
LaPorte, D. C., Wierman, B. M. & Storm, D. R. (1980) Biochemistry **19**, 3814–3819
Levine, B. A. & Dalgarno, D. C. (1983) Biochim. Biophys. Acta **726**, 187–204
Mani, R. S. & Kay, C. M. (1983) Biochemistry **22**, 3902–3907
Mani, R. S., Boyes, B. E. & Kay, C. M. (1982) Biochemistry **21**, 2607–2612
Margossian, S. S. & Stafford, W. F. (1982) J. Biol. Chem. **257**, 1160–1165
McCubbin, W. D. & Kay, C. M. (1973) Biochemistry **12**, 4228–4232
McDonald, J. R. & Walsh, M. P. (1985) Biochem. J. **232**, 559–567
Means, A. R., Tash, J. S. & Chafouleas, J. G. (1982) Physiol. Rev. **62**, 1–39
Murray, A. C. & Kay, C. M. (1972) Biochemistry **11**, 2622–2627
Seamon, K. B. (1980) Biochemistry **19**, 207–215
Siegel, L. M. & Monty, K. J. (1966) Biochim. Biophys. Acta **112**, 346–362
Tanaka, T. & Hidaka, H. (1980) J. Biol. Chem. **255**, 11078–11080
Walsh, M. P. & Hartshorne, D. J. (1982) in Calcium and Cell Function (Cheung, W. Y., ed.), vol. 3, pp. 223–269, Academic Press, New York
Walsh, M. P. & Hartshorne, D. J. (1983) in Biochemistry of Smooth Muscle (Stephens, N. L., ed.), vol. 2, pp. 1–84, CRC Press, Boca Raton, FL
Walsh, M. P., Stevens, F. C., Kuznicki, J. & Drabikowski, W. (1977) J. Biol. Chem. **252**, 7440–7443
Walsh, M. P., Stevens, F. C., Oikawa, K. & Kay, C. M. (1978) Biochemistry **17**, 3928–3930
Walsh, M. P., Stevens, F. C., Oikawa, K. & Kay, C. M. (1979) Can. J. Biochem. **57**, 267–278
Walsh, M. P., Valentine, K. A., Ngai, P. K., Carruthers, C. A. & Hollenberg, M. P. (1984) Biochem. J. **224**, 117–127
Wolff, D. J., Poirier, P. G., Brostrom, C. O. & Brostrom, M. A. (1977) J. Biol. Chem. **252**, 4108–4117

Received 15 May 1985/17 July 1985; accepted 7 August 1985