Purification and properties of a novel Ca^{2+} -binding protein (10.5 kDa) from Ehrlich-ascites-tumour cells*

Jacek KUŹNICKI and Anna FILIPEK

Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland

A novel Ca^{2+} -binding protein (CaBP) was identified in Ehrlich-ascites-tumour cells and purified to homogeneity. The molecular mass of this protein is about 10.5 kDa as estimated by polyacrylamide-gel electrophoresis in the presence of SDS. CaBP has two Ca^{2+} -binding sites that bind Ca^{2+} with a dissociation constant of about 3×10^{-6} M. Ca^{2+} binding to CaBP decreases its electrophoretic mobility in urea/polyacrylamide gels, changes its u.v. spectrum, increases the intrinsic tyrosine fluorescence intensity and strengthens hydrophobic interaction with the phenyl-Sepharose matrix.

INTRODUCTION

Ca^{2+} is involved in regulating a variety of cellular events such as enzyme activities and cell motility [for a review, see Rasmussen & Waisman (1982)]. These events are mediated through a class of homologous proteins that serve as Ca^{2+} receptors and are modulated by Ca^{2+} [see Kretsinger (1980) and Van Eldik *et al.* (1982) for reviews]. The proteins include calmodulin (reviewed by Manalan & Klee, 1984), troponin C, parvalbumin (reviewed by Heizmann, 1984), S-100 protein (reviewed by Donato, 1986), vitamin D-dependent Ca^{2+} -binding proteins (reviewed by Glenney *et al.*, 1982) and oncomodulin (reviewed by MacManus & Whitfield, 1983). All these proteins, except for calmodulin, are known to be tissue- or species-specific and to perform specific functions.

It has been shown that Ca^{2+} -sensitivity and the levels of some Ca^{2+} -binding proteins are altered in tumorigenic cells. For instance, Ca^{2+} , which is required for proliferation of cells cultured *in vitro*, has little or no influence on the proliferation of tumour cells (Whitfield *et al.*, 1979). Also, the concentration of calmodulin (reviewed by Veigl *et al.*, 1984) or S-100 protein (Kato *et al.*, 1985) increases in some tumours, whereas in other cells the synthesis of oncomodulin is initiated (MacManus & Whitfield, 1983). To understand the role of Ca^{2+} in the tumour cells, it seems necessary to identify and characterize the Ca^{2+} -binding proteins present in these cells. In the present work, a novel (10.5 kDa) Ca^{2+} -binding protein, hereafter called 'CaBP', was purified from Ehrlich-ascites-tumour cells and partially characterized. The purified protein exhibits the properties typical of the homologous Ca^{2+} -binding proteins which are modulated by Ca^{2+} . A preliminary account of this work has already been presented (Kuźnicki & Kowalczyk, 1986).

MATERIALS AND METHODS

Protein purification

Ehrlich-ascites-tumour cells were passaged in Swiss mice by intraperitoneal inoculation of about 5×10^7 cells,

and the cells were harvested after 10 days. All subsequent steps, except hydrophobic chromatography on the phenyl-Sepharose, were performed at 4 °C. The cells were washed three times with 0.9 % NaCl. The pellet of cells was homogenized in 5 vol. of deionized water in a glass/glass homogenizer. Stock solutions were added to the homogenate to obtain the final concentrations of the components as follows: 50 mM-KCl, 1 mM-2-mercaptoethanol, 1 mM-EDTA, 0.5 mM-PMSF, SBTI (20 µg/ml), Trasylol (Polfa) (50 units/ml) and 10 mM-Tris/HCl, pH 8.0. The homogenate was centrifuged for 1 h at 30000 g. The supernatant was applied to a DEAE-cellulose (DE-52, Whatman) column equilibrated with a buffer solution containing 1 mM-EDTA, 1 mM-2-mercaptoethanol, 0.25 mM-PMSF and 10 mM-Tris/HCl, pH 8.0. The unbound proteins were washed out with the same solution. The protein fraction containing CaBP was then eluted from the column with the solution containing 0.07 M-NaCl. To this fraction, CaCl_2 and NaCl were added at final concentrations of 1 mM and 0.5 M respectively, and the pH was adjusted to 7.5 with HCl. The solution obtained was applied to a phenyl-Sepharose (Pharmacia) column (2.6 cm × 20 cm) equilibrated with a solution containing 0.5 M-NaCl, 1 mM- CaCl_2 , 1 mM-2-mercaptoethanol, 0.25 mM-PMSF and 10 mM-Tris/HCl, pH 7.5, at room temperature. Most proteins were eluted from the phenyl-Sepharose with 50 % (v/v) ethylene glycol. CaBP was subsequently eluted with 2 mM-EGTA in 50 % ethylene glycol. The fraction obtained was concentrated by means of an Amicon PM 10 membrane and applied to a Sephadex G-75 (fine grade) column (2.5 cm × 100 cm) equilibrated with 10 mM- NH_4HCO_3 , pH 8.3. The peak of pure and desalted CaBP was concentrated by freeze-drying.

Bovine brain calmodulin and S-100 protein were prepared as described by Drabikowski *et al.* (1977). Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) and that in the presence of 6 M-urea and Tris/glycine buffer, pH 8.6, was performed as described by Perry *et al.* (1972).

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; SBTI, soybean trypsin inhibitor; CaBP, Ca^{2+} -binding protein from Ehrlich-ascites-tumour cells.

* This paper is dedicated to the memory of Professor M. Gabriela Sarzała-Drabikowska.

Ca²⁺ binding

Binding of Ca²⁺ to CaBP was measured directly by using the chromatographic method of Hummel & Dreyer (1962) and indirectly by measuring changes in fluorescence intensity of CaBP on titration with Ca²⁺. All buffer solutions were treated with Chelex-100 (Bio-Rad Laboratories). Only polypropylene containers and pipettes were used. They were rinsed several times in Chelex-treated water before use. Cuvettes for fluorimetric measurements were soaked for 16 h in ethanol and then rinsed several times with Chelex-treated water. Before the experiments CaBP was decalcified by addition of 1 mM-EGTA and 1 mM-EDTA and a subsequent gel filtration on a Sephadex G-25 column equilibrated with Chelex-treated 10 mM-Tris/HCl, pH 7.5. After this treatment the protein exhibited a tyrosine fluorescence intensity characteristic of a Ca²⁺-free protein. The Ca²⁺ present in the solutions as a contaminant was not considered in calculations, as it was assessed to be lower than 10⁻⁷ M. The free Ca²⁺ concentration in the solutions was estimated with a Ca²⁺-sensitive electrode (Fabczak, 1986). Calcium Lix exchanger (IE-202) in the electrode and calcium standards were from World Precision Instruments.

Direct Ca²⁺ binding to CaBP was measured by using a column of Sephadex G-25 (0.9 cm × 11 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, and various concentrations of ⁴⁵CaCl₂. The protein sample (0.6 mg) was applied in 0.2 ml of the above buffer containing the same ⁴⁵CaCl₂ concentration as that in the equilibration buffer. Bound Ca²⁺ was determined from the area of the radioactivity peak corresponding to the protein-bound ⁴⁵Ca²⁺.

Fluorescence titration of CaBP (0.38 mg/ml) was performed in 10 mM-Tris/HCl, pH 7.5. CaCl₂ (4.48 mM) was added with a Hamilton microlitre syringe to the apoprotein, and changes in tyrosine fluorescence intensity were monitored. Ca²⁺ bound to protein was calculated from the plots of changes in the fluorescence intensity versus the molar ratio of the added Ca²⁺ to the protein (see Fig. 2 below), with the *M_r* of CaBP assumed to be 10500. The Ca²⁺ binding constant was calculated from the equation:

$$\beta = \frac{1}{c^n} \times \frac{A/B}{(1 - A/B)^{n+1}}$$

where *n* is the number of Ca²⁺ ions bound, *A/B* is a ratio of changes of fluorescence intensity (see Fig. 2 below), and *c* is the total Ca²⁺ concentration. At *n* = 2, *β* is a product of the association constants for the two sites.

Spectroscopic measurements

The effect of Ca²⁺ on the u.v. spectrum of CaBP was examined with a Beckman DU-6 spectrophotometer. Fluorescence measurements were performed in Perkin-Elmer MPF-2L spectrofluorimeter equipped with a temperature-controlled cell holder, at 20 °C. Tyrosine fluorescence was monitored at 305 nm, with excitation at 280 nm.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Amino acid composition

Amino acid analysis was performed, after hydrolysis of CaBP in 6 M-HCl at 110 °C for 24 h, by using Biotronik LC 6000E analyser with Hewlett-Packard 3353A Laboratory Data System (the analysis was performed by Ms. A. Feldman in the Faculty of Chemistry, University of Bielefeld, Bielefeld, Germany).

RESULTS AND DISCUSSION

Purification of CaBP

The purification method described here consists of three rapid chromatographic steps. First, DEAE-cellulose ion-exchange chromatography at pH 8.0 was designed to obtain a protein fraction containing CaBP free of calmodulin. CaBP from Ehrlich-ascites-tumour cells was eluted with 0.07 M-NaCl, whereas calmodulin remained bound to the DEAE-cellulose. The second purification step led to separation of CaBP from the bulk of proteins by hydrophobic chromatography on a phenyl-Sephacrose column. In the presence of Ca²⁺ and 50% ethylene glycol, CaBP bound strongly to the hydrophobic matrix, whereas other proteins did not. Subsequent removal of Ca²⁺ from the medium resulted in elution of CaBP together with a few minor contaminants (Fig. 1a). The final purification of CaBP was achieved by gel filtration on a column of Sephadex G-75. About 20 mg of CaBP is routinely obtained from 100 g wet weight of cells.

Electrophoretic mobility of CaBP

At alkaline pH and in the presence of urea CaBP migrated as a single band, and its mobility was dependent on Ca²⁺ concentration. CaBP migrated faster in the absence of Ca²⁺ (EGTA added) than in the presence of this ion (Fig. 1a). Vitamin D-dependent Ca²⁺-binding protein from pig and mouse intestine had a similar electrophoretic behaviour (Hitchman *et al.*, 1973; Delorme *et al.*, 1982). The changes in electrophoretic mobilities distinguish the ascites protein from calmodulin (Fig. 1a), rabbit skeletal-muscle troponin C, carp muscle parvalbumin and bovine brain S-100 (not shown). In contrast with CaBP, calmodulin and troponin C have higher mobility in the presence of Ca²⁺ than in its absence, whereas the electrophoretic mobilities of S-100 protein as well as that of muscle parvalbumin in urea-containing gels are independent of Ca²⁺ concentration (Drabikowski *et al.*, 1977).

In the presence of SDS, CaBP from ascites cells migrated as a single band with a mobility similar to that of S-100 protein from bovine brain (Fig. 1b). Thus the molecular mass of CaBP from Ehrlich-ascites-tumour cells is about 10.5 kDa.

Amino acid composition

The possibility of a similarity between CaBP and other low-molecular-mass Ca²⁺-binding proteins (the intestinal vitamin D-dependent or the monomer of brain S-100 protein) can be eliminated by comparison of their amino acid compositions (Table 1). The amino acid composition of CaBP is also different from that calculated for 82 residues of the sequences of calmodulin or parvalbumin (Kretsinger, 1980). It seems therefore unlikely that CaBP is generated from high-molecular-mass proteins by proteolysis.

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of urea (a) or SDS (b)

Lanes 1a and 1b, protein fraction applied to the phenyl-Sephadex column; lanes 2a and 2b, protein fraction eluted from the phenyl-Sephadex with 50% ethylene glycol and EGTA; lanes 3a and 3b, calmodulin from bovine brain [1a, 2a and 3a, 0.1 mM-CaCl₂; 1b, 2b and 3b, 1.0 mM-EGTA]; lanes 4 and 6, CaBP from Ehrlich-ascites-tumour cells after Sephadex G-75 column chromatography (50 and 10 µg respectively); lane 5, calmodulin (CaM) and S-100 protein from bovine brain.

Table 1. Amino acid composition of CaBP and other Ca²⁺-binding proteins

Amino acid	Molar ratio	Proposed no. of residues per molecule						
		Vitamin D-dependent Ca ²⁺ -binding protein			Parvalbumin			
		CaBP	Rat*	Guinea pig†	Bovine S-100b protein‡	Rat oncomodulin§	Rat§	Rabbit
Aspartate	9.59	10	10	8	9	17	15	12
Threonine	2.01	2	7	2	3	5	5	5
Serine	3.40	3	7	7	5	11	11	8
Glutamate	12.91	13	23	16	19	18	9	12
Proline	ND¶		0		0	2	0	1
Glycine	5.57	6	4	3-4	4	6	9	9
Alanine	8.38	8	5	2-3	5	9	11	11
Cysteine	ND		0		0	1	0	0
Valine	3.66	4	3	4	6	2	5	5
Methionine	0.64	1	2	0	3	3	3	3
Isoleucine	5.05	5	3	2	4	6	6	6
Leucine	13.37	13	15	10	8	8	9	9
Tyrosine	0.88	1	3	1	1	2	0	0
Phenylalanine	2.56	3	8	6	7	8	8	9
Histidine	1.88	2	0	0	5	1	2	2
Lysine	9.45	9	18	11-12	8	7	15	16
Arginine	2.10	2	0	0	1	2	1	1
Total no. of residues . . .		82	101	79	90	108	109	109

* Delorme *et al.* (1982). † Wasserman *et al.* (1978). ‡ Marshak *et al.* (1981). § MacManus & Whitfield (1983). || Enfield *et al.* (1975). ¶ ND, not determined.

Ca²⁺ binding

Fluorescence spectroscopy of CaBP showed the absence of tryptophan emission and the predominance of tyrosine emission (result not shown). Ca²⁺-free protein exhibited 18% lower tyrosine fluorescence intensity than Ca²⁺-saturated protein. These changes in the fluorescence intensity were reversible.

Fig. 2 shows titration of decalcified CaBP with CaCl₂. The titration exhibited an end point at a Ca²⁺/protein ratio of 1.6 (1.7 in a parallel experiment), indicating the existence of two Ca²⁺-binding sites. After the maximum

level of fluorescence intensity was reached, 2 mM-EGTA (final concn.) was added to establish the lowest level of fluorescence intensity; it was identical with the fluorescence intensity of the decalcified protein. The apparent dissociation constant for Ca²⁺, calculated from the fluorescence titration curve (Fig. 2) with the assumption that the two sites bind Ca²⁺ with equal affinity, is 3 × 10⁻⁶ M.

The Hummel & Dreyer (1962) gel-filtration method was used to check directly the binding of Ca²⁺ to the purified protein. The measurements were performed at different ⁴⁵Ca²⁺ concentrations. Fig. 3 shows the protein

Fig. 2. Ca^{2+} binding measured by titration of the fluorescence intensity of CaBP with increasing concentrations of Ca^{2+}

The changes in fluorescence at 305 nm (excitation at 280 nm) were plotted against the molar ratio of added Ca^{2+} to CaBP (at 20 °C). The ratio of the changes in the fluorescence intensity (A/B) was used to calculate the association constant (see the Materials and methods section). The solvent was 10 mM-Tris/HCl, pH 7.5.

Fig. 3. Ca^{2+} binding to CaBP

CaBP (3.0 mg/ml) was subjected to gel filtration in the presence of 3×10^{-5} M- $^{45}\text{CaCl}_2$ /20 mM-Tris/HCl, pH 7.5. ○, Protein concentration; ●, radioactivity of $^{45}\text{CaCl}_2$.

and radioactivity elution profiles obtained at 3×10^{-5} M- $^{45}\text{CaCl}_2$. The bulk of the protein was eluted at the void volume of the column and coincided with the peak-radioactivity fractions. The amount of bound Ca^{2+} was calculated from the specific radioactivity and protein

concentration in the peak. In the experiment shown in Fig. 3, the value of 0.7 mol of Ca^{2+} /mol of protein was obtained. A similar value was obtained when 5×10^{-5} M- Ca^{2+} was used (results not shown). These results indicate that only one Ca^{2+} -binding site is revealed by the method of Hummel & Dreyer (1962) at the Ca^{2+} concentrations used. The second Ca^{2+} -binding site might have been overlooked, since at higher concentrations of Ca^{2+} the $^{45}\text{Ca}^{2+}$ bound to the protein was a small fraction of the total Ca^{2+} in order to permit its accurate determination. These results suggest the existence of two sites with different affinities for Ca^{2+} , one of which (the one with lower affinity) is overlooked by the method of Hummel & Dreyer (1962). A similar conclusion has been reached by Shadle *et al.* (1985) to explain the discrepancy between the results of the direct gel-filtration method, which revealed the presence of one Ca^{2+} -binding site, and the spectroscopic results, which indicated the existence of two Ca^{2+} -binding sites in protein II from liver. It has also been reported that the monomer of S-100 protein possesses two Ca^{2+} -binding sites with different structure and affinity for Ca^{2+} [reviewed by Donato (1986)].

Fig. 4. U.v. absorption spectrum of CaBP (4.4 mg/ml) in the presence of 1 mM-EGTA (a), and Ca^{2+} -induced u.v. difference spectrum of CaBP (b)

Protein (1 mg/ml) was in 20 mM-Tris/HCl, pH 7.5, and 0.1 mM- CaCl_2 . After recording the baseline (----), 1 mM-EGTA was added and the difference spectrum (—) was recorded.

Ca²⁺-induced conformational changes

The effect of Ca²⁺ on the conformation of CaBP from ascites cells was also studied by means of u.v. difference spectroscopy. Fig. 4(a) shows the u.v. absorption spectrum of the Ca²⁺-free protein. An absorption maximum at 278 nm and a shoulder at 285 nm are apparent. Fig. 4(b) shows the u.v. difference spectrum between the Ca²⁺-saturated and the Ca²⁺-free protein. In the absence of Ca²⁺, an increase in absorbance from 270 to 290 nm was observed, with a negative peak at 283 nm. Similar changes in the u.v. spectrum have been observed for porcine intestinal CaBP (Dorrington *et al.*, 1974), calmodulin (Klee, 1977) and liver Ca²⁺-binding protein II (Shadle *et al.*, 1985). These Ca²⁺-induced absorption changes are thought to indicate an increased exposure of the tyrosine residue(s) to the aqueous solvent in the presence of ligands. The u.v. difference spectrum and fluorescence measurements indicate that, on Ca²⁺ binding, conformational changes in CaBP take place near tyrosine residue(s).

Conclusions

In the present work we describe the physicochemical properties of a novel Ca²⁺-binding protein (10.5 kDa) from Ehrlich-ascites-tumour cells. It is an acidic protein with two Ca²⁺ binding sites, which seem to have different affinities for Ca²⁺. CaBP is modulated by Ca²⁺, as indicated by the changes in its u.v. absorption, fluorescence intensity and affinity to phenyl-Sepharose on Ca²⁺ binding. CaBP from ascites cells differs in its electrophoretic mobility from calmodulin, S-100 protein from brain and parvalbumin from muscle. It is also different from oncomodulin (J. P. MacManus, personal communication). CaBP from ascites cells closely resembles the homologous proteins modulated by Ca²⁺ in its lack of tryptophan, Ca²⁺-binding parameters and Ca²⁺-dependent changes of conformation. However, the similarity may be superficial, and the amino acid sequence of the ascites protein has to be established before such a conclusion can be reached.

The function of CaBP protein is as yet unknown, and further studies are needed in this area.

We thank Dr. J. P. MacManus (National Research Council of Canada) for showing that the Ca²⁺-binding protein described in this paper is different from oncomodulin. We thank Dr. S. Fabczak at the Nencki Institute of Experimental Biology for measuring Ca²⁺ concentrations with his Ca²⁺-sensitive electrodes, and Mrs. R. Klocke for typing the manuscript. We are grateful to Professor H. Strzelecka-Golaszewska and Ms. W. Leśniak for their comments on the manuscript, and to Ms. A. Feldmann from the University of Bielefeld for amino acid

analysis of CaBP. This work was supported by a grant from the Polish Academy of Sciences (CPBP 04.01).

REFERENCES

- Delorme, A. C., Danan, J. L., Ripoche, M. A. & Mathieu, H. (1982) *Biochem. J.* **205**, 49–57
- Donato, R. (1986) *Cell Calcium* **7**, 123–145
- Dorrington, K. J., Hui, A., Hofmann, T., Hitchman, A. J. W. & Harrison, J. E. (1974) *J. Biol. Chem.* **249**, 199–204
- Drabikowski, W., Kuźnicki, J. & Grabarek, Z. (1977) *Biochim. Biophys. Acta* **485**, 124–133
- Enfield, D. L., Ericsson, L. H., Blum, H. E., Fischer, E. H. & Neurath, H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1309–1313
- Fabczak, S. (1986) *Acta Protozool.* **25**, 315–324
- Glennay, J. R., Jr., Matsudaira, P. & Weber, K. (1982) in *Calcium and Cell Function* (Cheung, W. Y., ed.), pp. 356–380, Academic Press, New York
- Heizmann, C. W. (1984) *Experientia* **40**, 910–921
- Hitchman, A. J. W., Kerr, M. K. & Harrison, J. E. (1973) *Arch. Biochem. Biophys.* **155**, 221–227
- Hummel, J. P. & Dreyer, W. J. (1962) *Biochim. Biophys. Acta* **63**, 530–532
- Kato, K., Haimoto, H., Ariyoshi, Y., Horisawa, M., Washida, H. & Kimura, S. (1985) *Jpn. J. Cancer Res.* **76**, 856–862
- Klee, C. B. (1977) *Biochemistry* **16**, 1017–1024
- Kretsinger, R. H. (1980) *CRC Crit. Rev. Biochem.* **8**, 119–174
- Kuźnicki, J. & Kowalczyk, A. (1986) *Proc. Symp. Calcium Calcium-Binding Proteins, Crans-sur-Sierre* **24**
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. C. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacManus, J. P. & Whitfield, J. F. (1983) in *Calcium and Cell Function* (Cheung, W. Y., eds.), pp. 412–440, Academic Press, New York
- Manalan, A. S. & Klee, C. B. (1984) *Adv. Cyclic Nucleotide Res.* **18**, 227–278
- Marshak, D. R., Watterson, D. M. & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6793–6797
- Perry, S. V., Cole, H. A., Head, J. F. & Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 251–262
- Rasmussen, H. & Waisman, D. M. (1982) *Rev. Physiol. Biochem. Pharmacol.* **95**, 111–148
- Shadle, P. J., Gerke, V. & Weber, K. (1985) *J. Biol. Chem.* **260**, 16354–16360
- Van Eldik, L. J., Zendegui, J. G., Marshak, D. R. & Watterson, D. M. (1982) *Int. Rev. Cytol.* **77**, 1–61
- Veigl, M. L., Vanaman, T. C. & Sedwick, W. D. (1984) *Biochim. Biophys. Acta* **738**, 21–48
- Wasserman, R. H., Fullmer, C. S. & Taylor, A. N. (1978) in *Vitamin D* (Lawson, D. E. M., ed.), pp. 133–166, Academic Press, New York
- Whitfield, J. F., Boynton, A. L., MacManus, J. P., Sikorska, H. & Tsang, B. K. (1979) *Mol. Cell Biochem.* **27**, 155–179