Isolation and Characterization of a Novel Molecular Weight 11 000 Ca^{2+}-Binding Protein from Smooth Muscle†

Rajam S. Mani and Cyril M. Kay*

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

Received May 3, 1989; Revised Manuscript Received September 12, 1989

ABSTRACT: A new low molecular weight calcium-binding protein, designated as SMCaBP-11, has been isolated from chicken gizzard using a phenyl-Sepharose affinity column followed by ion-exchange and gel filtration chromatographies. The isolated protein was homogeneous by the criteria of gel electrophoresis in the absence and presence of sodium dodecyl sulfate ($NaDodSO_4$). Molecular weight studies by both sedimentation equilibrium in 6 M guanidine hydrochloride and 15% polyacrylamide–SDS gels indicated the subunit molecular weight to be 11 000, and since a molecular weight of 21 000 was obtained in native solvents, the protein exists as a dimer in benign medium. The amino acid composition of this protein is similar but distinct from other known low molecular weight Ca^{2+}-binding proteins. Ca^{2+}-binding assays using Arsenazo III (Sigma) indicated the protein to bind 2 mol of Ca^{2+}/subunit. In non-SDS gels, the protein moved faster in the presence of EDTA, suggesting that Ca^{2+} binding affects its mobility in a manner similar to other smooth muscle calcium-binding proteins such as calmodulin and 67-kDa calcimedin. Upon binding calcium, the protein underwent a conformational change as revealed by UV difference spectroscopy and circular dichroism studies in the aromatic and far-ultraviolet range. When the protein was excited at 280 nm, the tyrosine fluorescence emission maximum was centered at 306 nm. Ca^{2+} addition resulted in a nearly 15% decrease in intrinsic fluorescence intensity. Fluorescence titration with Ca^{2+} exhibited two classes of calcium-binding sites with K_d values of 0.2 and 80 μM, in agreement with UV difference spectral data. Like calmodulin and other related Ca^{2+}-binding proteins, this protein also exposes a hydrophobic site upon binding calcium which may indicate a physiological role for this protein in Ca^{2+}-mediated events in smooth muscle.

Physiological responses such as contraction, secretion, glycogenolysis, and mitogenesis in excitable cells are often preceded by an elevation of free calcium. These events are mediated through a class of homologous proteins that serve as calcium receptors and are modulated by Ca^{2+} [see reviews by Kretsinger (1980) and Van Eldik et al. (1982)]. Major proteins included in this group are calmodulin (Manalan & Klee, 1984), troponin C, parvalbumin (Heizmann, 1984), S-100 proteins (Donato, 1986; Mani & Kay, 1987), vitamin D dependent Ca^{2+}-binding proteins (Glenney et al., 1982), and oncomodulin (MacManus & Whitfield, 1983). These regulatory Ca^{2+}-binding proteins generally undergo a conformational change in the presence of Ca^{2+}. This calcium-induced conformational change enables them to interact with their target protein thereby affecting the properties of the target protein. For example, the binding of Ca^{2+} to calmodulin induces rather extensive conformational changes (Dedman et al., 1977; Klee 1977; Walsh et al., 1978; Seamon, 1980) including exposure of a hydrophobic site which is believed to be a sie of interaction with target proteins (LaPorte et al., 1980; Tanaka & Hidaka, 1980). This exposure of a hydrophobic site in the presence of Ca^{2+} has led to the use of Ca^{2+}-dependent hydrophobic interaction chromatography for purifying various calcium-binding proteins (Moore & Dedman, 1982). Such an approach was used in our laboratory for isolating 67-kDa calcimedin, a Ca^{2+}-binding protein present in chicken gizzard. During its isolation, we detected a novel low molecular weight Ca^{2+}-binding protein with a subunit molecular weight of 11 000. This protein, referred to as SMCaBP-11,[1] has been purified and characterized. The purified protein (SMCaBP-11) exhibits the properties typical of the homologous Ca^{2+}-binding proteins which are modulated by Ca^{2+}.

Materials and Methods

Protein Purification. About 350 g of chicken gizzard was homogenized in a Waring blender using 1 L of buffer consisting of 40 mM Tris, pH 7.5, 80 mM NaCl, 2 mM EDTA, 0.05% sodium azide, 1 mM PMSF, and 0.5 mL of aprotinin. The homogenate was centrifuged in a Beckman J2-21 centrifuge at 10 000 rpm for 75 min. Proteins in the supernatant were concentrated by ammonium sulfate addition to 90% saturation. Precipitated protein was dissolved in 40 mM Tris, pH 7.5, containing 80 mM NaCl and subjected to centrifugation at 14 000 rpm for 45 min. Free calcium concentration in the supernatant was adjusted to 2 mM. The supernatant in the presence of Ca^{2+} was applied to a phenyl-Sepharose column equilibrated with 80 mM NaCl, 2 mM $CaCl_2$, and 40 mM Tris, pH 7.5. Calcium-binding proteins were eluted from this column by using 4 mM EGTA. Eluted proteins were next applied to a DEAE-cellulose column in the presence of 20 mM imidazole, pH 6.2, and 1 mM EDTA. Calcium-binding proteins were eluted by using a linear NaCl gradient. Under these conditions, SMCaBP-11 eluted around 0.15 M NaCl in peak III as indicated in Figure 1. Isolated protein was essentially homogeneous (>90 pure). Peak III material when

† Supported by the Alberta Heart Foundation and the Medical Research Council of Canada.

[1] Abbreviations: SMCaBP-11, smooth muscle calcium-binding protein, M_r 11 000; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/90/0429-1398$02.50/0

analyzed by SDS–polyacrylamide gel electrophoresis revealed SMCaBP-11 as the major protein along with some 67-kDa calcimedin. These two proteins could easily be separated on an Ultragel AcA44 (LKB) gel filtration column equilibrated with 50 mM Tris, pH 7.5, containing 200 mM NaCl, 2 mM EDTA, and 0.05% NaN_3. The first major peak corresponded to 67-kDa calcimedin, and the protein isolated under peak II was a homogeneous preparation of SMCaBP-11. The major proteins in peaks IV and V from the DEAE-cellulose column were 67-kDa calcimedin and calmodulin.

Standard polyacrylamide gel electrophoresis was performed at pH 8.6 with Tris/glycine buffer as described by Schaub and Perry (1969). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and molecular weight determinations were carried out as described in one of our earlier publications (Mani & Kay, 1983). Amino acid analyses were performed on a Durrum D-500 amino acid analyzer (Dionex Corp.). Protein concentrations were established in the ultracentrifuge by employing the Rayleigh interference optical system, assuming 41 fringes equivalent to 10 mg/mL (Babul & Stellwagen, 1969). From a plot of the number of fringes versus optical density, a value of 6.8 was established as the extinction coefficient, $E^{1\%}_{1cm,277nm}$, for this protein in the absence of Ca^{2+}. Ultracentrifuge studies were routinely done at 20 °C in a Beckman Spinco Model E ultracentrifuge equipped with a photoelectric scanner, multiplex accessory, and high-intensity light source. The Rayleigh interference optical system was also employed. Low-speed sedimentation equilibrium runs were performed according to Chervenka (1969). A partial specific volume of 0.73 mL/g was assumed in native solvents, and a value of 0.72 mL/g was assumed in the guanidine hydrochloride medium.

UV absorption and UV difference spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer over the wavelength range 320–250 nm with 1-cm path-length cells. The spectra were measured at 25 °C with a Lauda thermoregulator connected to the sample compartment. To generate a UV difference spectrum, Ca^{2+} was added to the sample cell, and an equal volume of Chelex-100-treated water was added to the reference cell. Corrections were made for dilutions before the spectrum was plotted. Circular dichroism (CD) measurements were made on a Jasco J500C instrument fitted with a DP-500N data processor as described previously (Mani & Kay, 1983). Fluorescence spectra were obtained with a Perkin-Elmer Model MPF-44 spectrofluorometer, and all measurements were made at 20 °C. The instrument was operated in a ratio mode. The A_{277nm} of the sample was less than 0.1. For fluorescence titration experiments with Ca^{2+}, the concentration of free Ca^{2+} ions in solution was controlled by means of an EGTA-containing buffer and employing principles outlined by Perrin and Dempsey (1974).

Calcium-binding assays were carried out as described in an earlier paper on S-100b protein (Mani & Kay, 1986), using the relatively new Centricon 10 microconcentrators from Amicon as ultrafiltration devices to perform rapid flow dialysis as a means of monitoring Ca^{2+} binding to SMCaBP-11. SMCaBP-11 protein was incubated for 15 min with excess calcium and was then centrifuged in a Beckman Model L centrifuge for 30 min at 4000 rpm. Free Ca^{2+} in the supernatant (in this case, the solution centrifuged across the dialysis membrane) was analyzed by using the metallochromic indicator Arsenazo III (Sigma) according to Thomas (1982). SMCaBP-11 was initially dissolved in 25 mM HEPES buffer, pH 7.5. Protein (20–40 nmol) was placed in a prewashed Centricon 10 microconcentrator along with a known amount of Ca^{2+} incubated for 15 min and the solution centrifuged. The supernate was assayed for Ca^{2+} with Arsenazo III by using a 20 μM solution of dye in 0.1 M Tris-HCl, pH 7.5, and monitoring the Ca^{2+}-induced absorbance changes at 650 nm on a Perkin-Elmer Lambda 5 spectrophotometer. Standard curves (known amount of Ca^{2+} added versus A_{650}) were generated at the start and the conclusion of an experiment to ensure the reproducibility of the dye. From the amount of free Ca^{2+} found in the supernatant (from the standard curve), along with the total Ca^{2+} added and the amount of protein used, it was possible to calculate the amount of Ca^{2+} bound to the protein.

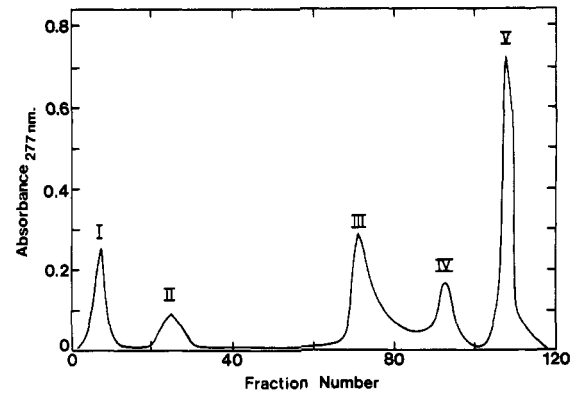

FIGURE 1: Elution profile of SMCaBP-11 from a DEAE-cellulose column. Solvent system used was 20 mM imidazole, pH 6.2, and 1 mM EDTA containing 1000 mL of a linear NaCl gradient from 0 to 0.25 M.

RESULTS

Calcium-binding proteins including SMCaBP-11, calcimedins (Moore & Dedman, 1982), and calmodulin were eluted from the phenyl-Sepharose column in the presence of EDTA. SMCaBP-11 was resolved from 67-kDa calcimedin and calmodulin on a DEAE-cellulose column using a linear NaCl gradient to 0.25 M. Under these conditions, SMCaBP-11 eluted around 0.15 M NaCl (peak III, Figure 1). The purity of the protein was greater than 90% and was further purified on an AcA44 Ultragel column (LKB). When the isolated SMCaBP-11 was run in 15% polyacrylamide–sodium dodecyl sulfate, it migrated with a molecular weight value of 11K and was homogeneous (Figure 2). The gels were calibrated with standard proteins using the Sigma gel kit protein samples. The mobility of the protein in non-SDS gels is also shown in Figure 2. The protein moved faster in the presence of EDTA. A decrease in mobility in the presence of Ca^{2+} could be due to a decrease in negative charge on the protein resulting from binding the cation. In this respect, SMCaBP-11 behaves similarly to calmodulin and 67-kDa calcimedin but different from muscle troponin C.

Ultracentrifugation. Low-speed sedimentation equilibrium studies were carried out under denaturing conditions in 6 M guanidine hydrochloride/0.1 M Tris, pH 7.5, and the observed molecular weight in this medium represents the minimum or subunit molecular weight. A plot of ln OD vs r^2 under denaturing conditions was linear, and the slope term yielded a molecular weight of 10 500 ± 500. The initial loading concentration for the particular run in Figure 3 was 0.64 mg/mL. The subunit molecular weight obtained from SDS–polyacrylamide gel electrophoresis is in excellent agreement with this value. Sedimentation equilibrium runs in benign medium consisting of 0.1 M Tris, pH 7.5, and 0.1 M NaCl yielded a molecular weight of 21 000 ± 500, suggesting that the protein exists as a dimer of 21 000 molecular weight in native solvents.

Table I: Amino Acid Composition of SMCaBP-11 and Other Ca^{2+}-Binding Proteins

	proposed no. of residues per molecule							
		vitamin D dependent Ca^{2+}-binding protein			bovine S-100b	rat	parvalbumin	
amino acid	SMCaBP-11	CaBP[a]	rat[b]	guinea pig[c]	protein[d]	oncomodulin[e]	rat[e]	rabbit[f]
aspartate	8.3	10	10	8	9	17	15	12
threonine	3.0	2		2	3	5	5	5
serine	3.8	3	7	7	5	11	11	8
glutamate	16.5	13	23	16	19	18	9	12
proline	2.5		0		0	2	0	1
glycine	9.8	6	4	3–4	4	6	9	9
alanine	10.5	8	5	2–3	5	9	11	11
cysteine	0		0		0	1	0	0
valine	3.7	4	3	4	6	2	5	5
methionine	2.3	1	2	0	3	3	3	3
isoleucine	4.0	5	3	2	4	6	6	6
leucine	11.5	13	15	10	8	8	9	9
tyrosine	4.3	1	3	1	1	2	0	0
phenylalanine	4.5	3	8	6	7	8	8	9
histidine	1.6	2	0	0	5	1	2	2
lysine	10.7	9	18	11–12	8	7	15	16
arginine	1.5	2	0	0	1	2	1	1
total no. of residues	100	82	101	79	90	108	109	109

[a] Kuznicki and Filipek (1987). [b] Delorme et al. (1982). [c] Wasserman et al. (1978). [d] Marshak et al. (1981). [e] MacManus & Whitfield (1983). [f] Enfield et al. (1975).

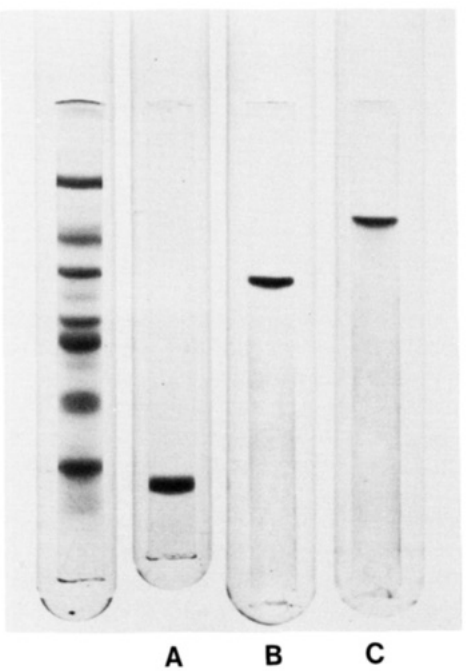

FIGURE 2: Electrophoresis of SMCaBP-11 in (A) 15% polyacrylamide–$NaDodSO_4$; (B and C) SMCaBP-11 in 12.5% polyacrylamide gels in Tris–glycine buffer, pH 8.6, in 1 mM EDTA and 1 mM Ca^{2+}, respectively.

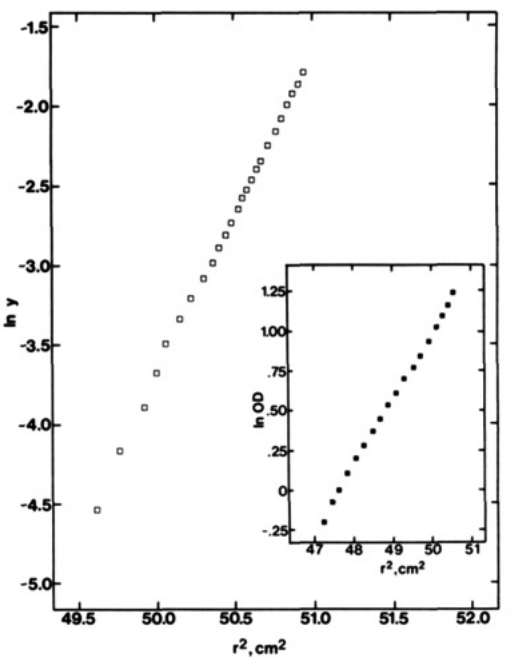

FIGURE 3: Typical plots of ln y versus r^2 plot for SMCaBP-11. The initial loading concentration was 0.84 mg/mL in 0.1 M Tris, pH 7.5, containing 0.1 M NaCl (□) and 0.64 mg/mL in 6 M Gdn-HCl/0.1 M Tris, pH 7.5 (■). The rotor speed was 40 000 rpm for the native solvent and 32 000 rpm for the 6 M Gdn-HCl medium at 20 °C.

Since sedimentation studies under denaturing conditions (6 M Gdn-HCl in the absence of any reducing agent) yielded a subunit molecular weight of 10 500, one may infer that the polypeptide chains in the protein are held together by noncovalent forces. In this respect, this protein behaves similarly to brain S-100 proteins which are also known to exist as dimers ($\beta\beta$ or $\alpha\beta$) of 21 000 molecular weight. However, the two proteins are very different on the basis of their amino acid compositions (see below) and spectral properties.

Ca^{2+} Binding. The number of bound Ca^{2+} ions per molecule of protein obtained from the direct calcium-binding assay using Arsenazo III were plotted against −log [free Ca^{2+}]. The protein bound 2 mol of calcium per subunit (Figure 4). The curve was fitted by the Hill equation describing the binding of ligand (Ca^{2+}) to the protein (Edsall & Gutfreund, 1983). Best fit was observed when the Hill coefficient (n) was 1.3 and the calculated apparent dissociation constant (K_d) for Ca^{2+} was 3×10^{-6} M. The observed K_d value probably represents a composite value for the two sites since the value of n obtained was greater than 1 (also see the fluorescence results below).

Amino Acid Composition. A comparison of the amino acid analysis of our preparation of SMCaBP-11 with other known low molecular mass Ca^{2+}-binding proteins is summarized in Table I. As can be noted, SMCaBP-11 is distinct from the proteins listed in the table especially when one considers Phe:Tyr ratios since other proteins have a low content of tyrosine with the result that the Phe:Tyr ratio is anywhere from 3 to 8 whereas SMCaBP-11 has a high content of tyrosine (4 residues) and a Phe:Tyr ratio close to 1. Among the proteins listed, the CaBP isolated from Ehrlich ascites tumor cells by

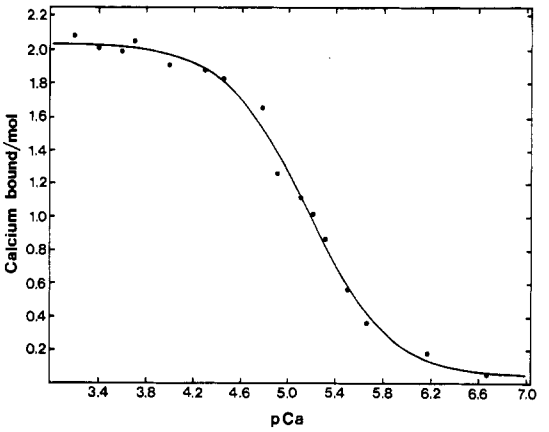

FIGURE 4: Ca^{2+} binding to SMCaBP-11. The experiment was carried out as described in the text. The solid circles represent the results from the binding assay. The solvent system used for the assay was 25 mM HEPES buffer, pH 7.5. The solid line represents the best fit of the data using the procedure described in the text.

Kuznicki and Filipek (1987) is the one that resembles more closely our protein. Even in this instance one can notice differences at the levels of methionine, tyrosine, and glycine residues suggesting that these two proteins may be similar but not identical. The most marked difference resides in the Phe:Tyr ratio which is 1 for SMCaBP-11, whereas for CaBP it is equivalent to 3. SMCaBP-11 has no sulfhydryl group(s) since the DTNB reaction, even in the presence of 6 M Gdn-HCl, gave no indication for any sulfhydryl group in the protein while the CaBP exists as a dimer of 21 000 molecular weight by forming an S–S bridge (Kuznicki, 1989; Filipek et al., 1989). In this respect also our protein differs from the CaBP which incidentally was found to be homologous to that of human calcyclin (Filipek et al., 1989). Dedman and his associates have purified antibodies for calcimedins, a set of four calcium-binding proteins that have been isolated from smooth muscle. These proteins are also referred to as calelectrins, and according to Südhof et al. (1988), 67-kDa calelectrin contains a duplication of the 4 repeat sequences of 68 residues found in lipocortins which are also called calpactins, suggesting that 67-kDa calelectrin has evolved by a series of gene duplication events; i.e., calelectrin and calpactins have evolved from a common ancestor. When tested, none of the calcimedin antibodies would recognize SMCaBP-11 (J. Dedman, personal communication), suggesting that SMCaBP-11 is not a fragment of any calcimedins or calpactins. The UV absorption spectrum of SMCaBP-11 is shown in Figure 5. The protein has no tryptophan since tryptophan-containing proteins generally will have a shoulder around 290 nm. In addition, the derivative spectrum clearly demonstrates that the protein has no tryptophan since tryptophan-containing proteins will exhibit a pronounced peak around the 292-nm region. The fine structure that one usually associates with calcium-binding proteins, like calmodulin, S-100b, and TN-C in the 250–270-nm region, is not noticeable, and this is due to the fact that the Phe to Tyr ratio is nearly 1 for SMCaBP-11 whereas for the other above-mentioned calcium-binding proteins this ratio is high; e.g., for S-100b, it is 7.

UV Difference Spectroscopy. The possible effect of Ca^{2+} on the tyrosine and phenylalanine aromatic groups was investigated by measuring UV difference spectra. Figure 6 shows the difference spectrum of SMCaBP-11 when 1 and 2 molar equiv of Ca^{2+} are added to the protein. The dominant difference peaks at 287 and 280 nm arise from the perturbation of one or more tyrosine residues. The sign of the tyrosyl difference peaks suggests that the chromophore is in a less

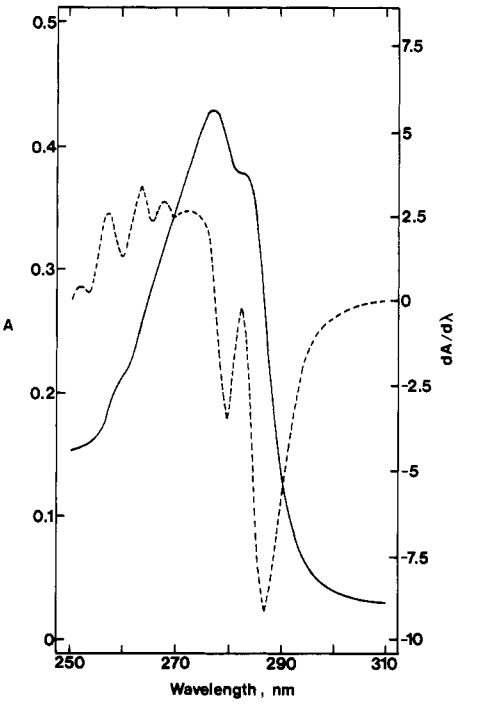

FIGURE 5: UV absorption (—) and derivative spectra (---) of SMCaBP-11 in 0.1 M Tris, pH 7.5 at 25 °C.

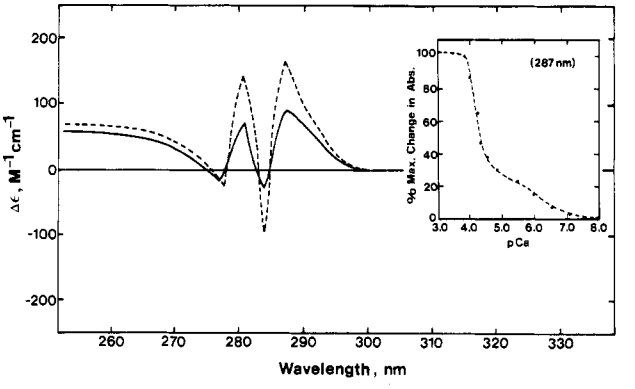

FIGURE 6: Change in molar extinction coefficient ($\Delta\epsilon$), obtained by UV difference spectroscopy, when SMCaBP-11 was mixed with 1 (solid line) or 2 (dashed line) molar equiv of Ca^{2+}/mol of protein. In both experiments, the concentration of SmCaBP-11 used was 0.4 mg/mL in 0.1 M Tris, pH 7.5, containing 0.1 mM EGTA. Insert: The changes in absorption (Abs) at 287 nm, measured at increasing free Ca^{2+} concentrations, are expressed as a percentage of the maximum change.

polar environment in the presence of Ca^{2+} (Donovan, 1969). The change in absorption of the protein at 287 nm as the protein was titrated with increasing Ca^{2+} is expressed as a percentage of the maximum change and plotted against −log [free Ca^{2+}] (insert Figure 6). From the titration data, it is possible to identify the presence of two classes of calcium-binding sites on the protein with K_d values of 8×10^{-7} and 6×10^{-5} M which are in good agreement with our fluorescence results (see below).

Circular Dichroism Studies. Typical far-UV CD spectra of SMCaBP-11 in 0.1 M Tris, pH 7.5, in the absence and presence of Ca^{2+} are shown in Figure 7. In the absence of Ca^{2+}, the $[\theta]_{222nm}$ is nearly −15 400 ± 300 deg·cm^2·dmol^{-1}, while the addition of Ca^{2+} causes a slight increase to −16 000 ± 300 deg·cm^2·dmol^{-1}, respectively. Analysis of the CD data according to the Chen et al. (1974) method indicates a decrease in apparent α-helical content from 54% to 49% in the presence of Ca^{2+}. The β-structure content increases from

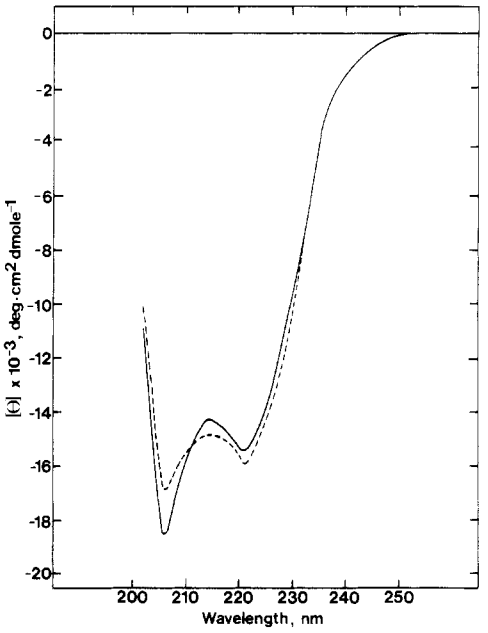

FIGURE 7: Far-ultraviolet CD spectra of SMCaBP-11 in (—) 0.1 M Tris, pH 7.5, and in (---) 0.1 M Tris, pH 7.5, containing 1 mM $CaCl_2$.

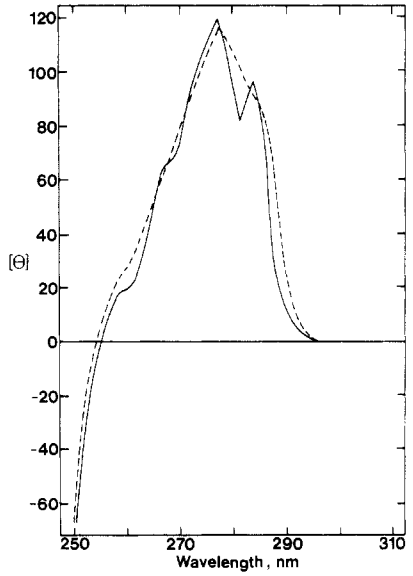

FIGURE 8: Aromatic CD spectra of SMCaBP-11 in (—) 0.1 M Tris, pH 7.5, and in (---) 0.1 M Tris, pH 7.5, containing 1 mM $CaCl_2$.

about 9% in the absence of Ca^{2+} to nearly 26% in the presence of Ca^{2+}, while the random structure decreases from about 38% in the apo state to 25% in the presence of Ca^{2+}, and this is indicated by the observed loss in the $[\theta]_{207nm}$ value in the presence of Ca^{2+}. The protein seems to acquire a more ordered secondary structure in the presence of Ca^{2+}.

Figure 8 reveals the effect of Ca^{2+} on the aromatic CD spectrum of SMCaBP-11. The ellipticity of the protein is positive between 260 and 300 nm. The two bands at 284 and 277 nm can be assigned to the tyrosine residues. It is obvious that tyrosine residues are affected by the addition of Ca^{2+} in the 280-nm region. In the presence of Ca^{2+}, we no longer observed the two well-resolved CD bands at 284 and 277 nm, indicating that Ca^{2+} addition has perturbed the microenvironment around the tyrosine residues. When we added 1 mM Mg^{2+} to SMCaBP-11, there was no noticeable change in the CD spectrum, suggesting that Mg^{2+} has no significant effect on the protein conformation or alternatively Mg^{2+} simply does not bind to this protein. In the far-UV CD region also, Mg^{2+}

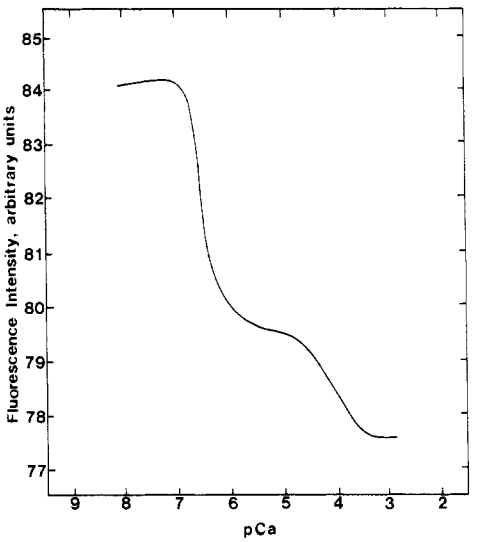

FIGURE 9: Plot of the fluorescence intensity at 306 nm as a function of the negative logarithm of free Ca^{2+} (pCa) concentration for SMCaBP-11 in 0.1 M Tris, pH 7.5, and 1 mM EGTA at 20 °C. The excitation wavelength was 280 nm, and the protein concentration was 2 μM.

had no pronounced effect on the secondary structure of the protein. The order of addition of the metal ions (Ca^{2+} and Mg^{2+}) had no significant effect on the final CD spectrum, implying that Ca^{2+} can bind to SMCaBP-11 in the presence of Mg^{2+} and induce the conformational change that was observed with the apoprotein. This observation is significant when one considers a physiological role for this protein since under in vivo conditions one would expect Mg^{2+} to be present.

Fluorescence Spectroscopy. Since the protein has only tyrosine and phenylalanine residues, the observed fluorescence spectrum will be that of tyrosine. Upon excitation at 280 nm, the emission maximum occurred at 306 nm. Addition of Ca^{2+} resulted in a nearly 15% decrease in the observed fluorescence intensity of 306 nm. A plot of the relative fluorescence intensity versus calcium added is shown in Figure 9, and from the fluorescence titration data, it is possible to identify the existence of two sets of calcium-binding sites on the protein with K_d values of 2×10^{-7} and 8×10^{-5} M. The solvent system employed for this titration consisted of 0.1 M Tris, pH 7.5, and 1 mM EGTA. Free Ca^{2+} concentration was calculated by using a computer program developed in Dr. Brian Sykes laboratory based on the original method of Perrin and Dempsey (1974).

Discussion

Calcium-binding proteins are known to bind to a hydrophobic matrix in a calcium-dependent manner, and this property was utilized in developing a scheme for isolating SMCaBP-11. The muscle homogenate after initial centrifugation was concentrated by ammonium sulfate precipitation to 90%. Proteins precipitated by 90% ammonium sulfate were applied to a phenyl-Sepharose column in the presence of Ca^{2+}. SMCaBP-11 along with other calcium-binding proteins was eluted from this column by using EGTA. SMCaBP-11 was further purified on a DEAE-cellulose column followed by gel filtration chromatography. The isolated isolated protein was homogeneous when tested by polyacrylamide–SDS gel electrophoresis. Sedimentation equilibrium studies in the ultracentrifuge suggest that this protein exists as a dimer of M_r 21 000 in native solvents like the S-100 system (Mani & Kay, 1987). Direct calcium-binding studies suggested the protein to bind 2 mol of Ca^{2+}/subunit. Fluorescence and UV dif-

ference Ca^{2+} titration indicated the presence of one high- and one low-affinity calcium-binding site on the protein. However, direct binding assays yielded only one K_d value (3×10^{-6} M) which conceivably represents an average value for the two sites; i.e., by this method, we are not able to distinguish between the two calcium-binding sites as their K_d values differ only slightly over an order of magnitude, or alternatively the observed difference may be attributed to different ionic conditions used in these experiments. The protein SMCaBP-11 after purification could still bind to a freshly poured phenyl-Sepharose column in 40 mM Tris, pH 7.5, containing 80 mM NaCl and 2 mM Ca^{2+}. It could be eluted from this column only in the presence of EGTA, suggesting that this proein also exposes a hydrophobic region in the presence of Ca^{2+} like other calcium-binding proteins belonging to the calmodulin family. We also used a hydrophobic fluorescent probe, TNS (2-*p*-toluidinylnaphthalene-6-sulfonate), which fluoresces weakly in polar solvents, but its intensity becomes enhanced in apolar solvents or when bound to a hydrophobic pocket on the protein (McClure & Edelman, 1966). TNS-labeled apoprotein when excited at 345 nm had an emission maximum at 445 nm. Addition of Ca^{2+} to TNS-labeled protein resulted in a 80% enhancement in fluorescence intensity, and this was accompanied by a blue shift of the emission maximum to 435 nm, implying that the probe in the presence of Ca^{2+} occupies a more hydrophobic environment; i.e., the protein exposes a hydrophobic region in the presence of calcium, a finding consistent with its ability to bind to a phenyl-Sepharose column. These results indicate that the secondary structure of the protein is influenced by Ca^{2+}. The CD studies reported herein have confirmed this idea. The apoprotein has some 53% α-helix, 9% β-pleated sheet, and 38% random coil, whereas the protein in the presence of Ca^{2+} has 49% α-helix, 26% β-structure, and 25% random coil.

Ca^{2+}-induced perturbations in the environment of tyrosine and phenylalanine residues as revealed by both near -UV CD and difference spectroscopy are certainly in line with previous observations on other calcium-binding proteins (Mani & Kay, 1987; Kay et al., 1987). Since the aromatic residues are red shifted, one can infer that these chromophores are in a more nonpolar environment in the presence of Ca^{2+}. Alternatively, the charge neutralization induced by Ca^{2+} might have perturbed the environment around these moieties in a similar fashion.

In conclusion, these spectral observations support the possibility that this novel Ca^{2+}-binding protein resembles calmodulin, S-100, and other calcium-binding proteins in undergoing a Ca^{2+}-induced conformational change. Since major Ca^{2+} receptors in cells are modulated by Ca^{2+}, one may infer that SMCaBP-11 which has a high affinity for calcium should also have a physiological role in Ca^{2+} mediated events in smooth muscle, in addition to calmodulin. Of interest in this connection is our finding that the average yield of SMCaBP-11 from this preparative method was 10 mg/350 g of wet tissue and this figure represents roughly 20% of the calmodulin content in chicken gizzard based on our procedure. Efforts are underway in our laboratory to identify the target protein for SMCaBP-11 as a first step in our approach to study its functional role in smooth muscle.

Acknowledgments

We thank A. Keri, K. Oikawa, and L. Hicks for their excellent technical assistance and M. Nattriss for performing the amino acid analyses.

Registry No. Ca, 7440-70-2.

References

Babul, G., & Stellwagen, E. (1969) *Anal. Biochem. 28*, 216–221.

Chen, Y., Yang, J. T., & Chan, 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.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977) *J. Biol. Chem. 252*, 8415–8422.

Delorme, A. C., Danam, J. L., Ripoche, M. A., & Mathiew, H. (1982) *Biochem. J. 205*, 49–57.

Donato, R. (1986) *Cell Calcium 7*, 123–145.

Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) p 101, Academic Press, New York.

Edsall, J. T., & Gutfreund, H. (1983) in *Biothermodynamics*, pp 157–207, Wiley, New York.

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.

Filipek, A., Heizmann, C. W., & Kuznicki, J. (1989) in *First European Symposium on Calcium Binding Proteins in Normal and Transformed Cells* (Pochet, R., Ed.) G8, Bruxelles, Belgium.

Glenney, 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.

Kay, C. M., McCubbin, W. D., & Sykes, B. D. (1987) *Biopolymers 26*, S123–S144.

Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.

Kretsinger, R. H. (1980) *CRC Crit. Rev. Biochem. 8*, 119–174.

Kuznicki, J. (1989) in *First European Symposium on Calcium Binding Proteins in Normal and Transformed Cells* (Pochet, R., Ed.) F3, Bruxelles, Belgium.

Kuznicki, J., & Filipek, A. (1987) *Biochem. J. 247*, 663–667.

LaPorte, D. D., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814–3819.

MacManus, J. P., & Whitfield, J. F. (1983) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) pp 412–490, Academic Press, New York.

Manalan, A. S., & Klee, C. B. (1984) *Adv. Cyclic Nucleotide Res. 18*, 227–278.

Mani, R. S., & Kay, C. M. (1983) *Biochemistry 22*, 3902–3907.

Mani, R. S., & Kay, C. M. (1986) *Biochem. J. 238*, 715–719.

Mani, R. S., & Kay, C. M. (1987) *Methods Enzymol. 139*, 168–187.

Marshak, D. R., Watterson, D. M., & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6793–6797.

McClure, W. O., & Edelman, G. M. (1966) *Biochemistry 5*, 1908–1919.

Moore, P. B., & Dedman, J. R. (1982) *J. Biol. Chem. 257*, 9663–9667.

Perrin, D. D., & Dempsey, B. (1974) in *Buffer for pH and Metal-ion Control* (Albert, A., Ed.) Chapman and Hall Ltd., London.

Schaub, M. C., & Perry, S. V. (1969) *Biochem. J. 115*, 993–1004.

Seamon, K. B. (1980) *Biochemistry 19*, 207–215.
Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078–11080.
Van Eldik, L. J., Zendegu, J. G., Marshak, D. R., & Watterson, D. M. (1982) *Int. Rev. Cytol. 77*, 1–61.
Walsh, M. P., Stevens, F. C., Oikawa, K., & Kay, C. M. (1978) *Biochemistry 17*, 3928–3930.
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.

Refined Crystal Structure of Calcium-Liganded Carp Parvalbumin 4.25 at 1.5-Å Resolution†

Vinod D. Kumar,‡ Lana Lee,§ and Brian F. P. Edwards*,‡

Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201, and Department of Chemistry and Biochemistry, University of Windsor, Windsor, Canada N9B 3P4

Received June 6, 1989; Revised Manuscript Received October 9, 1989

ABSTRACT: The crystal structure of carp parvalbumin (pI = 4.25) has been refined by restrained least-squares analysis employing X-ray diffractometer data to 1.5-Å resolution. The final residual for 12 653 reflections between 10 and 1.5 Å with $I(hkl) > 2\sigma(I)$ is 0.215. A total of 74 solvent molecules were included in the least-squares analysis. The root mean square deviation from ideality of bond lengths is 0.024 Å. The model has a root mean square difference of 0.59 Å from the positions of the main-chain atoms in a previously reported structure [Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 201–228], which was refined by difference Fourier syntheses using data collected by film to 1.9 Å. Although the overall features of the two models are very similar, there are significant differences in the amino-terminal region, which was extensively refit, and in the number of oxygen atoms liganding calcium in the CD and EF sites, which increased from six to seven in the CD site and decreased from eight to seven in the EF site.

Parvalbumins are small, calcium-binding proteins that are found primarily in the muscle tissue of vertebrates. Their properties include acidic isoelectric points, amino acid compositions rich in phenylalanine and alanine, and binding sites for 2 mol of calcium/mol of protein with dissociation constants of 10^{-7}–10^{-9} M [reviewed in Kretsinger (1980)]. Calcium-binding parvalbumin was first identified in frog muscle by Deuticke (1934) and further characterized by Hamoir (1951). Parvalbumins have now been isolated from the muscles of a wide variety of fish such as carp (Konusu et al., 1965), hake (Pechère et al., 1971), pike (Rao & Geraday, 1973), and other vertebrates such as frog (Pechère et al., 1973) and chicken (Heizmann & Strehler, 1979). A parvalbumin has also recently been identified in the γ-aminobutyric acid containing cells of the rat cerebral cortex (Celio, 1986). Besides carp 4.25 parvalbumin, crystal structures have been reported for *Opsanus tau* parvalbumin (Kahn et al., 1985) and pike 4.1 parvalbumin (Declercq et al., 1988).

The physiological role of parvalbumins has remained obscure for a long time, since these proteins are not involved in glycolysis or osmotic regulation, nor do they serve any known nutritional role. However, several inferences have been made on the basis of their location and strong metal-binding capacities. For example, parvalbumins are associated with only certain types of muscles such as fast nerve impulse activated skeletal muscles, which suggests they are not part of the contractile machinery but are instead connected with the calcium-dependent regulation of this type of muscle. For example, mouse mutants deficient in parvalbumin exhibit tetanic contractures after the cessation of motorneuron stimulation (Stuhfauth et al., 1984). In the rat, the parvalbumin gene has promoter sequences that are homologous to conserved sequences in the promoter region of myosin light chain, suggesting a common mechanism for regulation (Berchtold et al., 1987).

There is also evidence that the function of parvalbumins is not limited to muscle cells alone. Parvalbumin has been localized specifically in γ-aminobutyric acid containing cells in the brain (Celio, 1986), and chicken thymic hormone, which is very similar in its physical and chemical properties to mammalian thymosins and thymopoietins, has been identified as a parvalbumin (Brewer et al., 1989). This latter paper is the first instance of an unequivocal, specific function for a parvalbumin and suggests that parvalbumins have a role in development of the immune system.

Five parvalbumins have been isolated from carp muscle with isoelectric points of <3.9, 3.95, 4.25, 4.37, and 4.47 (Pechère et al., 1971). They have been labeled as parvalbumins 1, 2, 3, 5a, and 5b, respectively (Pechère et al., 1971). Another notation (Coffee et al., 1974), which originated in the order of elution from an anion-exchange column, is parvalbumin A_1 (pI = 4.47), A_2 (pI = 4.37), B (pI = 4.25), C (pI = 3.95), and D (pI < 3.9). We have followed the practice of Declercq et al. (1988) and identified the parvalbumins by their isoelectric points. Carp 4.25 parvalbumin was sequenced by Coffee and Bradshaw (1973). The protein contains 108 amino acid residues, an acetylated amino terminus, and high amino acid compositions of alanine (20%), phenylalanine (10%), and lysine (10%). The three-dimensional crystal structure was determined by Kretsinger and Nockolds (1973) to 1.9-Å resolution by the multiple isomorphous replacement method. The structure was subsequently refined by Moews and Kretsinger (1975), at the same resolution by a real space refinement procedure of Diamond (1971). Their crystal structure analysis

† This work was supported in part by NIH Grants GM 33192 and RR 02945, a grant from NSERC, and a grant-in-aid from the WSU Center for Molecular Biology.
‡ Wayne State University School of Medicine.
§ University of Windsor.

0006-2960/90/0429-1404$02.50/0