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CALELECTRINS ARE A UBIQUITOUS FAMILY OF Ca^{2+}-BINDING PROTEINS PURIFIED BY Ca^{2+}-DEPENDENT HYDROPHOBIC AFFINITY CHROMATOGRAPHY BY A MECHANISM DISTINCT FROM THAT OF CALMODULIN

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SUMMARY: The calelectrins, a heterogeneous group of three new Ca^{2+}-binding proteins of M_r 67 000, 35 000 and 32 500, copurify with calmodulin during Ca^{2+}-dependent hydrophobic affinity chromatography (Südhof et al., Biochemistry, in press, 1984). This property is exploited for the rapid purification of all three calelectrins including for the first time the M_r 35 000, from commercially available acetone powders from several bovine tissues (heart, liver, brain, pancreas and testis). The nature of the Ca^{2+}-dependent interaction of the calelectrins with hydrophobic affinity matrices has been investigated. As with calmodulin, the Ca^{2+}-binding sites of all three purified calelectrins can be probed with Tb^{3+} which binds to them in a stoichiometric, saturable and Ca^{2+}-displaceable manner. However, using several hydrophobic fluorescence probes which bind to the proteins, contrary to calmodulin no Ca^{2+}-dependent exposure of hydrophobic sites could be detected in any of the three purified proteins. Therefore the Ca^{2+}-dependent purification of the calelectrins on hydrophobic affinity columns seems not to involve the surface exposure of hydrophobic sites and the calelectrins have in this respect little similarity to calmodulin.

Calcium is a universal second messenger in animal and plant cells whose action is dependent on the presence of Ca^{2+}-binding proteins; it is therefore of interest to characterize these proteins especially when they are evolutionarily conserved and

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Abbreviations. 1,8-ANS, 1-anilinonapthalene-8-sulphonic acid; NPN, N-phenyl-1-naphthylamine; 2,6-TNS, 2-(p-toluidinyl)naphthalene-6-sulphonic acid; Tris, Tris(hydroxymethyl)aminomethane.

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widely distributed. Calelectrin (1-5) may be such a protein: it was originally isolated from Torpedo marmorata as a Ca^{2+}-dependent membrane-binding protein of M_r 34 000 (1); it was shown to be evolutionarily conserved from Torpedo to man (2) and to be associated with vesicular structures in animal cells (3) where it might be involved in Ca^{2+}-dependent membrane traffic (4). We recently isolated two Ca^{2+}-binding proteins from bovine liver, brain and adrenal medulla which cross-react immunologically with Torpedo calelectrin, one of M_r 32 500 very strongly, the other (M_r 67 000) weakly, and identified a third M_r 35 000 protein in these tissues which also cross-reacts (5). These proteins were shown to bind Ca^{2+} at 1 uM Ca^{2+}, to bind to membranes in a Ca^{2+}-dependent manner at micromolar Ca^{2+} concentrations and to possess mutual limited cross-reactivity. The mammalian calelectrins coelute with calmodulin from hydrophobic affinity columns in the presence of EGTA and we therefore decided to investigate whether they have calmodulin-like properties as has been suggested (6) for proteins called 'calcimedins' (7) that coelute with calmodulin from hydrophobic affinity columns. I also report here a fast purification procedure of all three proteins from several tissues based on commercially available acetone powders.

METHODS

Preparative procedures

Acetone powders (obtained from Sigma, Munich, FRG) were extracted with 0.1 M NaCl, 50 mM Tris pH 7.4, 2 mM PMSF and 2 mM EGTA [15 $ml.(g \text{ of acetone powder})^{-1}$] for 1 h at 4°C, with 2 x 10^5 $units.ml^{-1}$ Trasylol (obtained from Bayer, Leverkusen) additionally for pancreas acetone powders. Extracts were centrifuged for 30 min at 40 000 g_{max}. Ca^{2+}-dependent hydrophobic affinity chromatography on phenyl-Sepharose, gel filtration and column chromatofocusing were performed as described (5). SDS-PAGE and immuno blotting procedures were done as reported (2). Protein concentrations were determined according to Bradford (8).

Fluorescence measurements

These were done in a Perkin-Elmer MPF4 fluorescence spectrophotometer in water-jacketted cuvette holders kept at 25°C. (1) Solutions of the three purified proteins at different concentrations were titrated with Tb^{3+} (99.9%, Aldrich, Nettetal, FRG) by small sequential additions, and the fluorescence was monitored at 285 nm excitation and 545 nm emission wavelengths with a 430 nm emission cutoff filter. Tb^{3+} protein complexes were also characterized spectrally, and the Ca^{2+}- and Mg^{2+}-displaceability of Tb^{3+} were investigated both by adding these metals at near saturation with Tb^{3+} and by performing titrations in their presence and absence. (2) The interaction of the hydrophobic fluorescence probes NPN, 1,8-ANS and 2,6-TNS (obtained from Molecular Probes, El Paso, USA) with the three calelectrins and with calmodulin was investigated as a function of Ca^{2+}, spectra being taken of the probes at low concentrations with or without the respective proteins in the presence and absence of Ca^{2+}. All spectra reported here are uncorrected except for background.

RESULTS AND DISCUSSION

Like calmodulin and the S 100 proteins, the calelectrins are Ca^{2+}-binding proteins. They can be specifically purified by Ca^{2+}-dependent hydrophobic affinity chromatography (5), suggesting that they have properties like calmodulin. Therefore it seemed desirable to investigate the extent of the similarity between these proteins. As shown in Figs. 1 and 2, the calelectrins can be purified together with calmodulin from bovine tissue acetone powder extracts by Ca^{2+}-dependent hydrophobic affinity chromatography as effectively as from fresh tissue extracts. Commercially available acetone powders are convenient starting materials for protein isolation (9,10). Under our conditions, the calelectrins constituted the major components of the protein fraction that specifically bound to phenyl-Sepharose in the presence of Ca^{2+} and was eluted in the presence of EGTA, and the calelectrins can be easily purified to near homogeneity by column chromatofocusing or gel filtration from the EGTA-eluent, including for the first time the M_r 35 000 protein (Fig. 1). Similar results to liver and testis were obtained with bovine brain, heart and pancreas acetone powders, and the

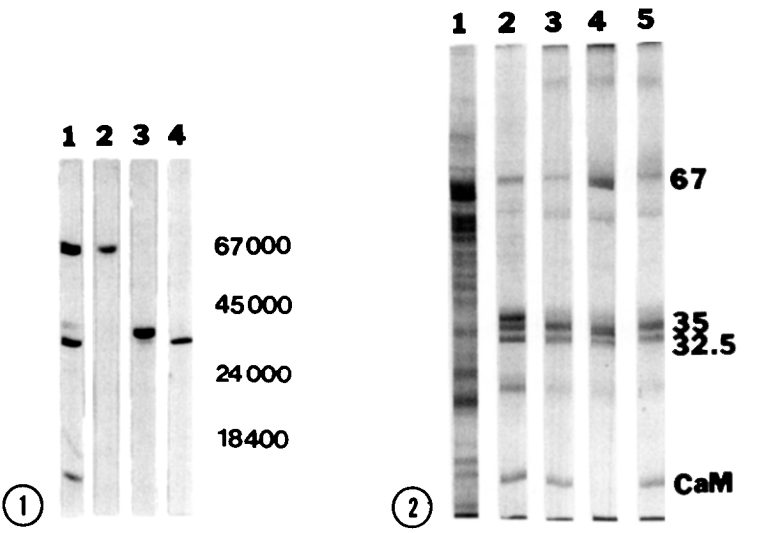

Fig. 1. Analysis of purified mammalian calelectrins on 15% SDS polyacrylamide gels. Lane 1 shows the Phenyl-Sepharose EGTA eluent from bovine liver from which the mammalian calelectrins were purified by chromatofocusing. Lane 2 shows the M_r 67 000, lane 3 the M_r 35 000 and lane 4 the M_r 32 500 calelectrin. Numbers to the right give the mobilities of the molecular weight markers bovine serum albumin (M_r 67 000), ovalabumin (M_r 35 000), trypsin (M_r 24 000) and -lactoglobulin (M_r 18 400).

Fig. 2. Ca^{2+}-dependent hydrophobic affinity chromatography of bovine testis acetone powder extract and the Ca^{2+}-dependent electrophoretic mobilities of the proteins in the EGTA eluent. The lanes show 10% SDS polyacrylamide gels of: 1, total bovine testis acetone powder extract; 2, Phenyl-Sepharose EGTA eluent from 1, run in the presence of mercaptoethanol without additions; 3, EGTA eluent run without mercaptoethanol in the presence of 10 mM EGTA; 4, EGTA eluent without mercaptoethanol but with 50 mM Ca^{2+}; 5, EGTA eluent without mercaptoethanol but with 50 mM Mg^{2+}. While there is a great change in the mobility of calmodulin (CaM), as a function of added Ca^{2+}, there is little change in the mobility of the calelectrins (67, M_r 67 000; 35, M_r 35 000, 32.5, M_r 32 500).

identity of the proteins was confirmed by immunoblotting (data not shown).

Proteins purified from acetone powder bound to membranes in a Ca^{+}-dependent manner were indistinguishable from that of proteins purified from fresh tissues (5), indicating that they retained their properties during acetone extraction. Some Ca^{2+}-binding proteins, like calmodulin, show a Ca^{2+}-dependent change in electrophoretic mobility. However, this is not so for the calelectrins (Fig. 2). Besides Ca^{2+}, calmodulin binds Tb^{3+} and

other metal ions (11, 12), which can be used as probes for the Ca^{2+}-binding sites. The same is true for the calelectrins. Spectra of Tb^{3+} show a several hundredfold increase in the specific Tb^{3+}-fluorescence in the presence of any of the three calelectrins (Fig. 3). The excitation spectra of the Tb^{3+}-calelectrin complexes show a maximum at 285 nm for all three proteins, a wavelength at which there is no absorption band for Tb^{3+} suggesting energy transfer from an aromatic amino acid of the protein to bound Tb^{3+}. The excitation maximum of the Tb^{3+} calelectrin complexes indicates tryptophan as the donor fluorophor; it also constitutes the main excitation peak of the proteins' intrinsic fluorescence. Addition of Ca^{2+}, and, to a lesser extent, Mg^{2+}, leads to Tb^{3+}-displacement (Fig. 3). Fluorescence

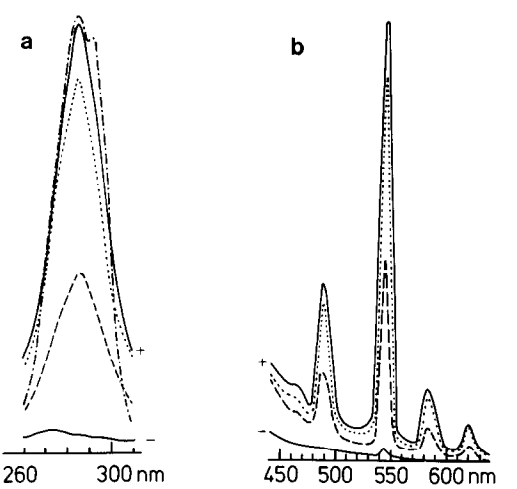

Fig. 3. (a) Excitation and (b) emission spectra of 0.24 mM Tb^{3+} in the presence (+) or absence (-) of 32 ug.ml^{-1} of M$_r$ 35 000 bovine liver calelectrin (continuous lines) and spectra of the same Tb^{3+} calelectrin complexes after the addition of 62 mM Mg^{2+} (dotted line) or 62 mM Ca^{2+} (dashed line). Addition of calelectrin to Tb^{3+} solutions leads to a several hundredfold increase in the Tb^{3+} luminescence which can be partially reversed by the addition of Mg^{2+} or Ca^{2+}. Figure (a) also shows an excitation spectrum of the native protein (dots and dashes) whose maximum is the same as that of the Tb^{3+} calelectrin complex. Spectra were taken in 0.1 mM KCl, 50 mM HEPES pH 7.4 with 4 nm slits. Tb^{3+} calelectrin excitation spectra were recorded at an emission wavelength of 545 nm with a 430 nm cutoff filter, the protein excitation spectra at 330 nm and the Tb^{3+} emission spectra at 285 nm excitation with a 430 nm emission cutoff filter. Very similar results were obtained with the other two calelectrins.

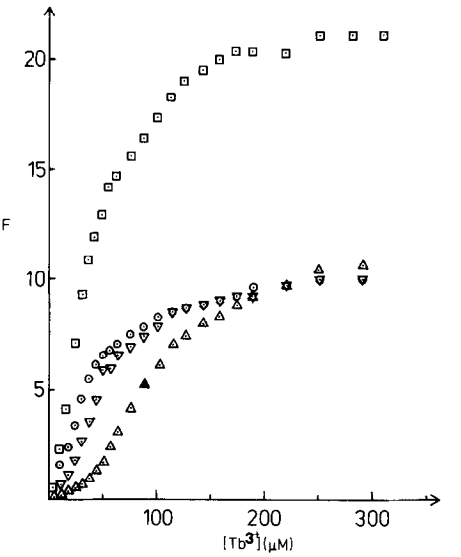

Fig. 4. Titration of the M_r 67 000 bovine liver calelectrin with Tb^{3+}. Solutions contained 0.18 $mg.ml^{-1}$ (squares) or 0.09 $mg.ml^{-1}$ of M_r 67 000 calelectrin (circles and triangles) in 0.1 M KCl, 50 mM HEPES pH 7.4 with 33 mM Mg^{2+} (inverted triangles) or 33 mM Ca^{2+} (triangles) additionally. The titrations demonstrate that the binding of Tb^{3+} to calelectrins is stoichiometric, saturable and Ca^{2+}-displaceable. Similar results were obtained with the other two calelectrins. The relative fluorescence (F) (arbitrary units) was monitored at excitation and emission wavelengths of 285 nm and 545 nm with a 430 nm emission cutoff filter.

titrations of calelectrin solutions with Tb^{3+} show that the binding of Tb^{3+} to the calelectrins is linear at low Tb^{3+} concentrations, stoichiometric and saturable (Fig. 4). I have not been able to calculate the exact ratio of mols of Tb^{3+} bound per mol of calelectrin, but it seems to be rather high (> 7). Fig. 4 also demonstrates that the Tb^{3+}-binding sites are Ca^{2+}-displaceable and, to a lesser extent, Mg^{2+}-displaceable.

It has been shown by the use of hydrophobic fluorescent probes that Ca^{2+}-binding by calmodulin leads to the exposure of hydrophobic sites (13), and this has been identified as the probable mechanism whereby calmodulin binds to hydrophobic matrices. However, using the same fluorescent probes (1,8-ANS; 2,6-TNS; and NPN), I could detect no significant Ca^{2+}-dependent change in the quantum yield in the presence of any of the

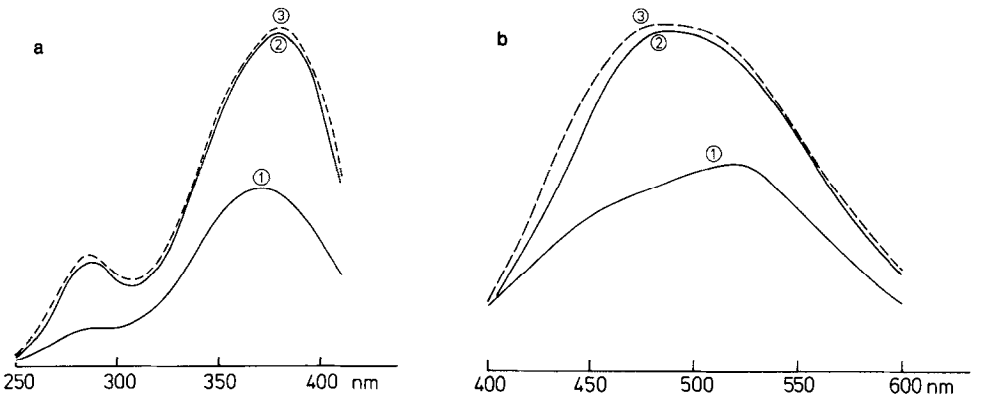

Fig. 5. (a) Excitation and (b) emission spectra of 13 uM 1,8-ANS without bovine liver calelectrin (spectrum 1) and with 66 ug.ml^{-1} of M_r 67 000 calelectrin (2,3) in the presence of 3.3 mM EGTA (2) or of 6.5 mM Ca^{2+} (3). Spectra were taken of solutions in 0.2 M MOPS pH 6.8, 50 mM KCl at excitation and emission wavelengths of 370 nm and 480 nm with 4 nm slits and emission cutoff filters of 430 nm (excitation spectra) or 390 nm (emission spectra). The spectra demonstrate a moderate fluorescence increase of the hydrophobic fluorescent probe 1,8-ANS in the presence of calelectrin. This fluorescence increase is Ca^{2+}-independent. Identical results were obtained with all three calelectrins (M_r 32 500, 35 000 and 67 000) with 1,8-ANS with two other hydrophobic probes (2,6-TNS and NPN).

purified calelectrins, although they all caused moderate, Ca^{2+}-independent probe fluorescence increases. This together with evidence for energy transfer from the aromatic amino acids of the proteins to the fluorescence probes indicate that the probes bind to the proteins (shown for the M_r 67 000 and 1,8-ANS in Fig. 5). Parallel experiments with calmodulin, however, showed an approximately 10-fold change in the fluorescence as function of Ca^{2+}.

I conclude that the calelectrins, like calmodulin, are Ca^{2+}-binding proteins whose Ca^{2+}-binding sites can be probed by Tb^{3+} and which bind to phenyl-Sepharose in a Ca^{2+}-dependent manner. However, they appear to have a high number of Ca^{2+}-binding sites per protein, and do not expose hydrophobic sites on their surface as a function of Ca^{2+}. Therefore the nature of their Ca^{2+}-dependent binding to phenyl-Sepharose must

be different from calmodulin's, and they seem to belong to a different class of Ca^{2+}-binding protein.

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