Purification of cloned trypanosomal calmodulin and preliminary NMR studies

P. J. SWEENEY* and J. M. WALKER

Division of Biological Sciences, Hatfield Polytechnic, College Lane, Hatfield, Hertfordshire AL10 9AB (U.K.)

and

D. G. REID and N. ELSHOURBAGY

Department of Chemistry, SmithKline Beecham Pharmaceuticals Ltd., The Frythe, Welwyn, Hertfordshire AL6 9AR (U.K.)

ABSTRACT

Cloned trypanosomal calmodulin was expressed in *Escherichia coli* and purified to homogeneity using hydrophobic interaction chromatography on phenyl-Sepharose. The purified protein was subjected to NMR analysis which allows detailed changes to be observed when, firstly, calcium, and secondly, the drug calmidazolium bind. These spectral changes are the result of conformational changes in the protein and proximity effects due to the drug.

INTRODUCTION

Calmodulin is a 17 000-dalton, multifunctional, universal regulator of calcium functions, which is present in all eukaryotic cells. The protein detects free calcium and translates changes in calcium concentrations into altered states of metabolism [1]. Calcium-bound calmodulin interacts with and regulates a large number of enzymes, particularly those of nucleotide phosphate transfer and hydrolysis [2].

Calmodulin has been purified to homogeneity from a wide variety of sources, including vertebrates, invertebrates, plants and protozoans [3,4]. The size, abundance, heat stability and ability of the calcium-bound protein to bind drugs and target proteins have provided the basis for a number of isolation procedures [5,6]. NMR studies on human and bovine calmodulins have yielded much information on the conformational transitions associated with calcium binding and drug interactions [7].

We report here the purification of cloned trypanosomal calmodulin using hydrophobic interaction chromatography. Calmodulin is a highly conserved protein. However, the trypanosomal protein contains 22 amino acid substitutions when compared to the human protein. Substitutions at positions 77 and 79 are unique to trypanosomal calmodulin and are in a region thought to be involved in drug and target protein interaction.

The purpose of these investigations is to study drug interactions with the trypanosomal protein and eventually compare assignments of human and trypanosomal calmodulins with a view to the design of trypanosomal calmodulin-specific drugs. We therefore also report preliminary NMR studies on the trypanosomal protein.

MATERIALS AND METHODS

E. coli strain AR58, containing the cDNA clone of trypanosomal calmodulin inserted into the expression vector pMG/Nco, was cultured in 25 250-ml conical flasks, each containing 100 ml of media, at 30°C, with shaking. The medium was a minimal bacterial medium, containing the following: 5 g/l sodium acetate, 2.5 g/l ammonium chloride, 1.9 g/l disodium hydrogen phosphate, 0.75 g/l potassium dihydrogen phosphate, 0.13 g/l sodium sulphate, 0.05 g/l magnesium sulphate, 0.2 ml LB broth and 0.05 ml trace element solution. When the cultures reached an absorbance of 0.4–0.6 at 550 nm, the flasks were transferred to a 42°C shaking water bath for 2 h to induce expression of calmodulin. The cells were then recovered by centrifugation, washed in 40 mM Tris–HCl, pH 7.5, resuspended in 250 ml of 50 mM Tris–HCl (pH 7.5), 1 mM mercaptoethanol, 1 mM EDTA (buffer 1), disrupted by sonication for 6 min, and the solution was clarified by centrifugation at 15 000 g for 15 min.

Calmodulin was purified from the clarified supernatant according to a method based on that described by Gopalakrishna and Anderson [8]. The soluble *E. coli* extract was passed through a phenyl-Sepharose (Pharmacia) column (14 cm × 1.6 cm I.D.), equilibrated with buffer 1, and the unbound fraction collected and made 5 mM in CaCl$_2$. It was then applied to a second phenyl-Sepharose column (14 cm × 1.6 cm I.D.), equilibrated with buffer 2 (50 mM Tris–HCl, pH 7.5, 1 mM mercaptoethanol, 0.1 mM CaCl$_2$) and the column washed with 15 column volumes of buffer 2, followed by 3 column volumes of buffer 3 (buffer 2 containing 0.5 M NaCl). The bound calmodulin was then eluted by washing the column with buffer 1. This fraction was made 50 mM in EDTA to remove calcium and the calcium free protein was then passed through a G-25 Sephadex (Pharmacia) column (45 cm × 1.6 cm I.D.), equilibrated with 100 mM ammonium bicarbonate, to remove EDTA, the calmodulin fractions were collected, pooled, freeze-dried and stored at −70°C.

Sodium dodecyl (lauryl) sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [9]. N-Terminal sequencing was determined by sample application to an Applied Biosystems 477 pulsed liquid phase sequencer, with a 120A phenylthiohydantoin analyser for detection (Applied Biosystems).

Proton NMR spectra were acquired at 37°C and 360 MHz on a Bruker AM360 spectrometer. A 25-mg sample of trypanosomal calmodulin was dissolved in 0.4 ml ^{2}H$_2$O (Aldrich), which was 0.1 M and 0.05 M with respect to potassium chloride and potassium phosphate and adjusted to pH 7.0. ^{43}CaCl$_2$ was used for the calcium titration experiments and calmidazolium was the compound chosen to perform the drug titration experiments. Calmidazolium (R24571) (Roche) was dissolved in deuterated methanol (Aldrich). One thousand scans were collected at each titration point and resolution enhancement was achieved using a convolution difference procedure (LB = 2.5) followed by exponential multiplication (LB = 1).
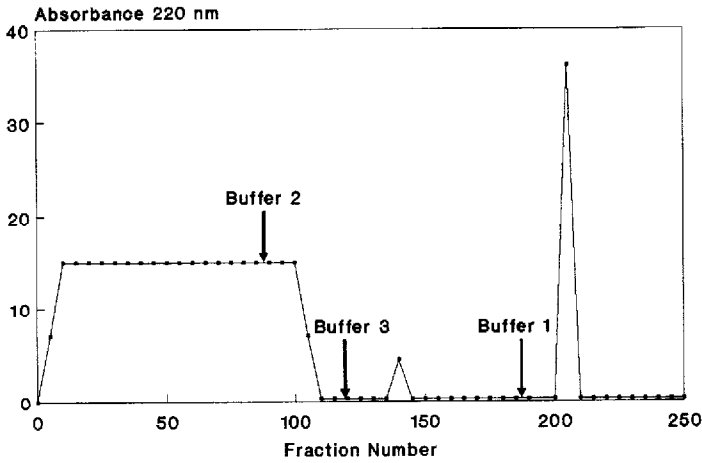
Fig. 1. Phenyl-Sepharose chromatography elution profile for trypanosomal calmodulin (second chromatography, see Results and Discussion).
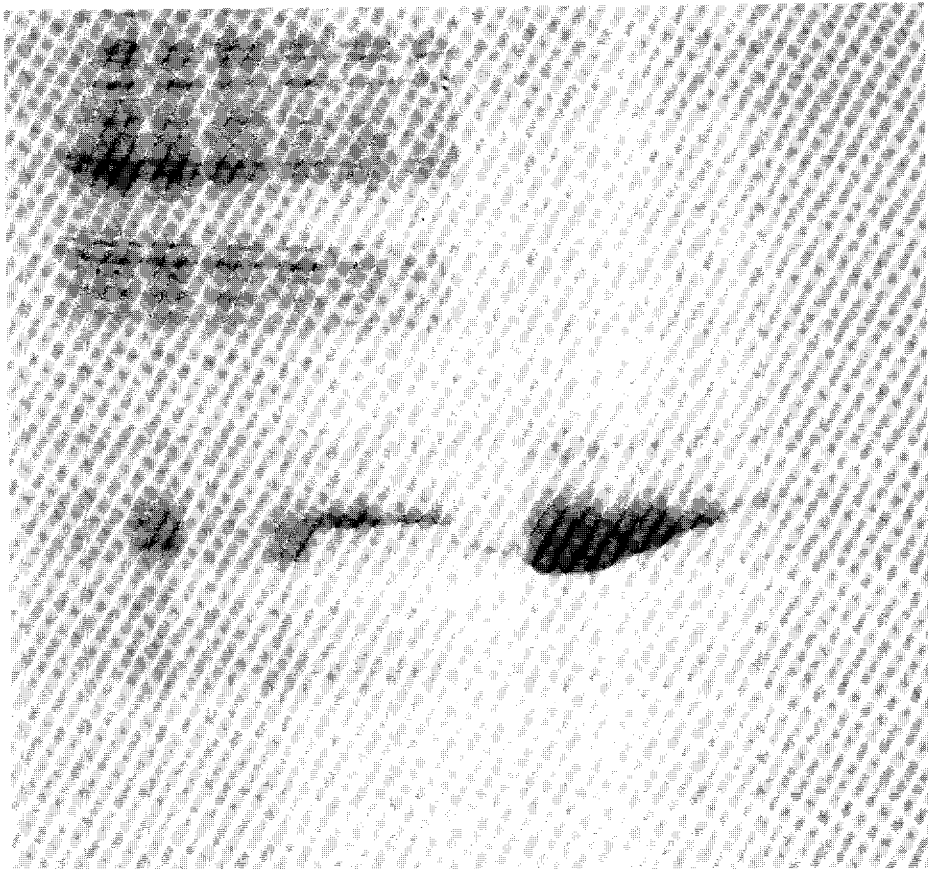

Fig. 2. SDS-PAGE analysis of *E. coli* extracts and purified trypanosomal calmodulin. From left to right, lanes 1,3: *E. coli* extract at point of induction, lanes 2,4: *E. coli* extract 12 h after induction, lanes 5,6: 12-h extracts in 1 m*M* EDTA (showing altered electrophoretic mobility of calmodulin in the absence of calcium), lanes 7–11: purified calmodulin fractions from column.

RESULTS AND DISCUSSION

Calmodulin was purified to homogeneity at a yield of 12–13 mg/l culture. The elution profile is illustrated in Fig. 1. The protein migrated as a single band on SDS-PAGE (Fig. 2) and N-terminal sequencing revealed a single N terminus. The first fifteen residues were identified by sequencing and agreed with the published sequence (single-letter code for amino acids): A–D–Q–L–S–N–E–Q–I–S–E–F–K–E–A.
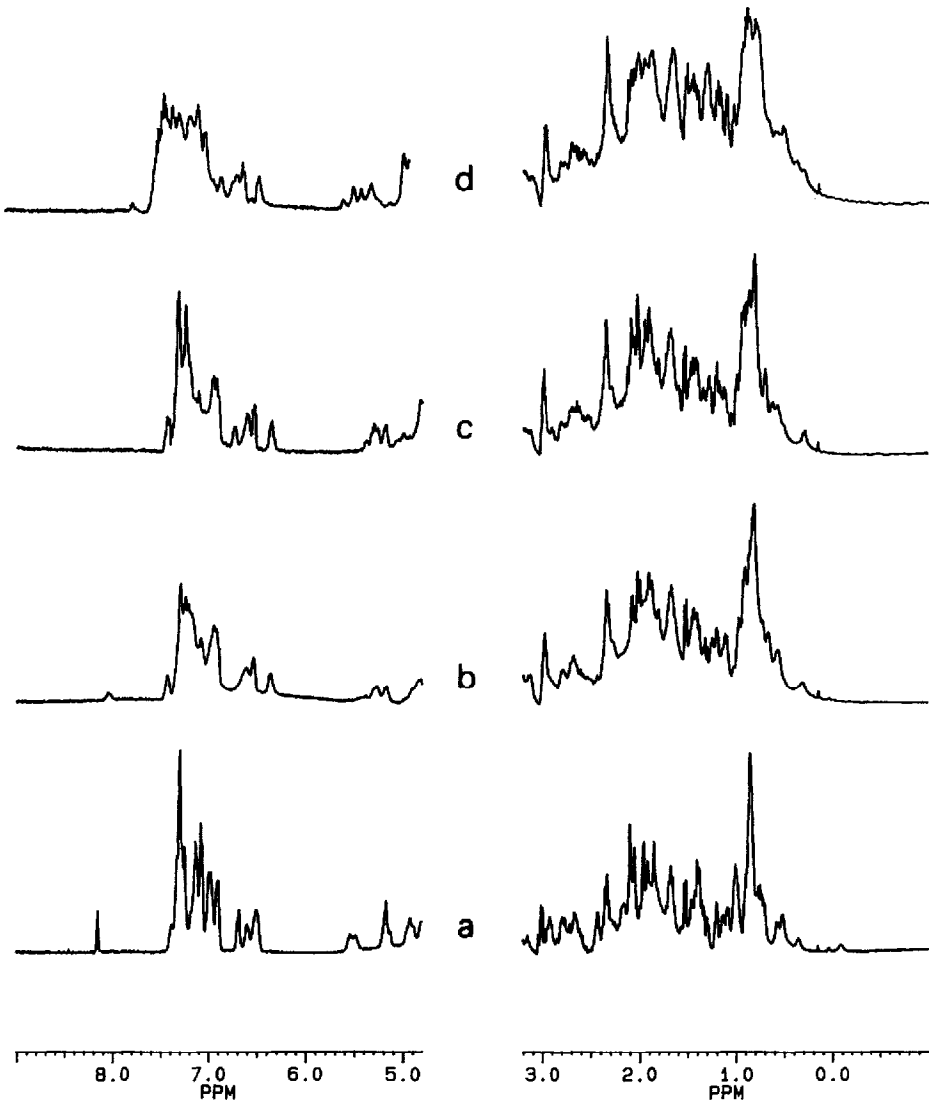

Fig. 3. NMR spectra of aromatic (4.5–9.0 ppm) and aliphatic (−1.0–3.0 ppm) regions of trypanosomal calmodulin. (a) 25 mg of calcium-free calmodulin, (b) 2:1 calcium–calmodulin complex, (c) 4:1 calcium–calmodulin complex, (d) 1:1 calmidazolium–calcium-saturated calmodulin complex.

The binding of calcium takes place in two distinct phases, corresponding to binding the two high-affinity, and the two low-affinity sites, respectively. As reported for bovine calmodulin [10], these two phases are characterized by slow exchange (discrete signals observed for Ca^{2+}-free and Ca^{2+}-bound protein) and fast exchange (time-averaged single resonances observed for both forms of the protein) on the NMR time scale, corresponding to successive occupation of the two high-affinity and two low-affinity sites. As shown in Fig. 3a, b and c, the interaction produces major conformational changes which are reflected in dramatic differences in the proton NMR spectra at each stage of the titration. Further changes accompany drug binding (Fig. 3d), corresponding, firstly, to the appearance of drug signals in the 6.5 to 7.5 ppm spectral region, and, secondly, to chemical shift and line-width changes in signals from the protein. The latter reflect both the effects of the drug on residues close to its binding site, and long range drug-induced conformational changes. The appearance of the aromatic region of trypanosomal calmodulin is very similar to that of the bovine protein, with the exception of the absence of the doublet corresponding to tyrosine, which may be accounted for by the Y-99–F-99 change in the former. However, spectral changes induced by the addition of one equivalent of calmidazolium are quite different from those observed in bovine calmodulin [11], although the compound still binds with high affinity, reflected in the slow exchange nature of most of the changes.

We are currently analysing the nature of the drug binding site by nuclear Overhauser effect experiments, assisted by selective and total deuteration of the protein, to allow drug signals to be observed in isolation, and assigned unambiguously.

REFERENCES

1 C. B. Klee and T. C. Vanaman, *Adv. Protein Chem.*, 35 (1982) 213.
2 P. Harris, in J. R. Jeter, I. L. Cameron, G. M. Madilla and A. M. Zimmerman (Editors), *Cell Cycle Regulation*, Academic Press, New York, 1978, p. 75.
3 Y. M. Lin, *Mol. Cell. Biochem.*, 45 (1982) 101.
4 L. Ruben, C. Egwuagu and C. L. Patton, *Biochim. Biophys. Acta*, 758 (1983) 104.
5 D. L. Newton, M. H. Krinks, J. B. Kaufman, J. Shilaoch and G. B. Klee, *Prep. Biochem.*, 18 (1988) 247.
6 G. A. Jamieson, Jr. and T. C. Vanaman, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1048.
7 J. S. Evans, B. A. Levine, R. J. P. Williams and M. R. Wormald, in P. Cohen and C. B. Klee (Editors), *Molecular Aspects of Cellular Regulation*, Vol. 5, Elsevier, Amsterdam, 1988, p. 57.
8 R. Gopalakrishna and W. B. Anderson, *Biochem. Biophys. Res. Commun.*, 104 (1982) 830.
9 U. K. Laemmli, *Nature (London)*, 277 (1970) 680.
10 K. B. Seamon, *Biochemistry*, 19 (1980) 207.
11 D. G. Reid, L. K. MacLachlan, K. Gajjar, M. Voyle, R. J. King and P. J. England, *J. Biol. Chem.*, 265 (1990) 9744.