IDENTIFICATION AND PURIFICATION OF A PHENOTHIAZINE BINDING FRAGMENT FROM BOVINE BRAIN CALMODULIN

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1. Introduction

Calmodulin is a small, acidic, intracellular calcium-binding protein which has been shown to influence the activity of a number of enzymic and structural systems in a calcium dependent manner (review [1]). The protein, which is apparently ubiquitous in eukaryotes, has been implicated as one of the principal intracellular targets for the calcium released intracellularly following various external stimuli. Of the various probes used to study the role of calmodulin one of the more useful has been the class of anti-psychotic drugs the phenothiazines which are able to bind to calmodulin in the presence of calcium, and block its modulating activity [2]. The specificity of the interaction of the various phenothiazines with calmodulin and the pharmacological significance of the interaction have been the subject of debate [3–5].

Here we have attempted to identify sites of interaction of phenothiazines on calmodulin using affinity chromatography of cyanogen bromide fragments of the molecule on fluphenazine–Sepharose.

2. Materials and methods

Fluphenazine was kindly given by Squibb and Sons (Princeton NJ). Other chemicals and reagents were laboratory reagent grade obtained through local suppliers. Bovine brains were obtained from Trelegan (Cambridge MA).

2.1. *Calmodulin preparation*

A calmodulin containing fraction was obtained from bovine brain by ion-exchange chromatography on DEAE-cellulose essentially as in [6]. The material eluting from the column at ~0.3–0.35 M NaCl in 20 mM Tris–HCl, 15 mM 2-mercaptoethanol, 1 mM EDTA (pH 7.8) was adjusted to a total concentration of 2 mM $CaCl_2$ by addition of 1/500th vol. 1 M $CaCl_2$ to give ~1 mM free Ca^{2+} ion. The pool was then applied directly to a column (2.5 × 20 cm) of fluphenazine–Sepharose (prepared as in [7]) equilibrated in 50 mM imidazole, 200 mM NaCl, 1 mM $CaCl_2$, 15 mM 2-mercaptoethanol (pH 7.2). Unbound material was washed from the column with 500 ml of this buffer, and the calmodulin then eluted with 300 ml of the same buffer but containing 5 mM EGTA in place of $CaCl_2$. Fractions (9 ml) were collected and the eluate monitored for calmodulin by alkaline urea slab-gel electrophoresis of samples from every tube. The calmodulin containing eluate was pooled, dialyzed against 2 changes of 50 vol. each of 2 mM Tris–HCl 15 mM 2-mercaptoethanol (pH 7.8) and lyophilized.

Calmodulin prepared by this method was single banded on SDS and alkaline urea gel electrophoresis and had full biological activity as judged by activation of calmodulin-dependent phosphodiesterase. The whole preparative routine takes <5 days, including dialysis and lyophilization, and yields ~110 mg calmodulin/kg brain. The purified calmodulin contains none of the 7000 M_r phenothiazine-binding protein described in [8].

2.2. *Cyanogen bromide cleavage*

Lyophilized calmodulin (5 mg) were dissolved in 0.5 ml 70% formic acid. Cleavage was begun by adding 10 μl of a 1 g/ml solution of CNBr in acetonitrile. The solution was incubated at 25°C for 24 h after which it was evaporated to dryness under a stream of nitrogen. The dried residue was resuspended in 200 μl H_2O and 1 M ammonium hydroxide solution added until the

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residue completely dissolved. The solution was again evaporated to dryness under a stream of N_2.

2.3. *Chromatography of CNBr fragments on fluphenazine–Sepharose*

The dried residue of CNBr fragments was redissolved in 2 ml 50 mM imidazole, 200 mM NaCl, 1 mM $CaCl_2$, 15 mM 2-mercaptoethanol (pH 7.2) and applied to a column (1.5 × 7 cm) of fluphenazine-Sepharose equilibrated in the same buffer. Unbound material was washed from the column with 200 ml buffer. The column was then eluted with 50 ml of the same buffer containing 5 mM EGTA in place of calcium. Fractions (5 ml) were collected throughout sample application, washing and elution. The eluate was monitored by running 16% acrylamide alkaline urea gel electrophoresis of samples from each tube.

2.4. *Phosphodiesterase assay*

Bovine brain, calmodulin-activatable, phosphodiesterase, prepared as in [6], was assayed using the method in [9]. Enzymic activity was determined at 37°C in 5 μM cyclic AMP, 0.1 mM $CaCl_2$ and in the presence or absence of calmodulin or the fluphenazine binding fragment.

2.5. *Electrophoresis*

SDS gel electrophoresis was carried out using 15% polyacrylamide gels and the buffer system in [10].

Alkaline gel electrophoresis was carried out using 16% polyacrylamide gels containing 6 M urea and a continuous buffer system of 25 mM Tris–80 mM glycine (pH 8.6) as in [11].

2.6. *Amino acid analysis*

Amino acid analysis was performed on samples hydrolyzed in 6 N HCl for 24 h at 110°C. Analyses were determined using a Beckman 119C1 analyzer with 126 data system. A column (0.6 × 24 cm) of W3 resin was used with the Beckman buffer system for collagen.

2.7. *Sequence analysis*

The sequence of the N-terminal end of the phenothiazine-binding fragment was determined by automated Edman degradation using a Beckman 890C sequencer equipped with a cold trap and a modification of program 121078 with 0.25 M Quadrol and a combined S_1 and S_2 wash. Phenylthiohydantoin derivatives of amino acids were determined by high-pressure liquid chromatography.

3. Results

Cyanogen bromide fragmentation of calmodulin produces peptides which can be visualized by electrophoresis on 16% polyacrylamide alkaline urea gel electrophoresis (fig.1a). This band pattern, obtained consistently, is characteristic for cyanogen bromide fragments of bovine brain calmodulin. When the fragments are applied to a column of fluphenazine–Sepharose in the presence of calcium the unbound material shows one band less than the characteristic pattern on electrophoresis (fig.1b). If the column is eluted with buffer containing 5 mM EGTA in place of calcium then the eluate is found to contain a peptide which migrates at a position corresponding to the band missing from the unbound material (fig.1c). This peptide migrates as a single band on SDS gel electrophoresis having an app. M_r-value close to that of bovine lung aprotinin (M_r 6500) (fig.1e).

The amino acid sequence of the N-terminal end of the fragment corresponds to the region of calmodulin extending from residue 77 (table 1). The total amino acid analysis of the fragment is shown in table 2. Comparison of this analysis with the analyses of the individual cyanogen bromide fragments, as determined in [12] shows no similarity. However, a sum of the analyses of equimolar amounts of CNBr 1A and CNBr 1B, using the designations in [12], closely corresponds to the values for the fluphenazine-binding fragment

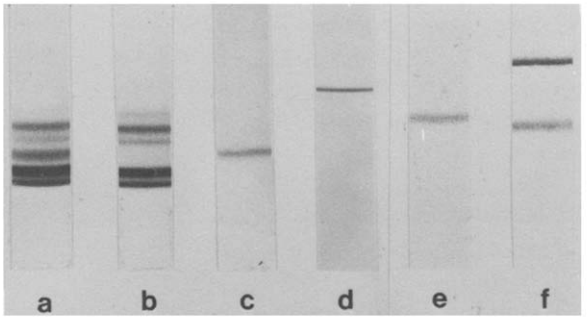

Fig.1. Alkaline urea and SDS gels of bovine brain calmodulin and its cyanogen bromide fragments: (a–d) 16% acrylamide alkaline urea gels; (e,f) 15% acrylamide SDS gels; (a) 50 μg cyanogen bromide fragments before application to fluphenazine–Sepharose column; (b) 50 μg unbound cyanogen bromide fragments from fluphenazine–Sepharose column; (c) 5 μg cyanogen bromide fragment eluted from fluphenazine–Sepharose column by EGTA; (d) 5 μg bovine brain calmodulin; (e) as (c) but on SDS gel; (f) 5 μg bovine brain calmodulin plus 5 μg bovine lung aprotinin (M_r 6500).

Table 1
N-terminal sequence of the phenothiazine binding fragment of calmodulin determined by automated Edman degradation

Amino acid	Residue number in calmodulin sequence	Step of fragment sequencing	Yield (nmol)
Lys	77	1	12.2
Asp	78	2	11.0
Thr	79	3	1.3
Asp	80	4	7.6
Ser[a]	81	5	n.d.
Glu	82	6	9.8
Glu	83	7	13.4
Glu	84	8	16.1
Ile	85	9	20.4
Arg	86	10	6.8

[a] Yields of this residue were insufficient to enable adequate determination

(table 2). These two fragments are contiguous in the sequence of calmodulin (residues 77–124) and are connected by a Met_{109}–Thr_{110} bond. Met–Thr bonds have been reported as often undergoing incomplete cleavage, the methionine being converted into homoserine without breaking the peptide bond [13]. Since the fluphenazine binding peptide migrates as a single band on electrophoresis we tentatively designate the fragment as corresponding to 1A–1B in which the Met–Thr bond has not been cleaved. The existence of such a fragment in cyanogen bromide digests of calmodulin has been reported in [12,14,15].

The fluphenazine binding fragment does not activate the calmodulin-dependent phosphodiesterase of bovine brain when included in enzyme assays in amounts in 10-fold molar excess over the amount of native calmodulin required to produce maximal activation.

4. Summary and discussion

We have isolated a cyanogen bromide fragment from bovine brain calmodulin which is able to bind to fluphenazine–Sepharose in a calcium-dependent manner. We have tentatively designated this fragment as corresponding to CNBr 1A-CNBr 1B in which the Met_{109}–Thr_{110} bond has not been cleaved. The calculated M_r of this peptide (5500) is slightly lower than might be judged from its mobility on SDS gel electrophoresis in comparison to bovine lung aprotinin, however, anomalies in the relationship between the

Table 2
Amino acid analysis of fluphenazine binding fragment and CNBr 1A–1B

	Fluphenazine binding fragment[a]	CNBr 1A–1B[b] (residues 77–124)
Asx	8.4	8
Thr	2.5	3
Ser	1.3	2
Glx	9.8	9
Pro	0	0
Gly	3.8	3
Ala	3.3	3
Val	3.6	3
Ileu	2.9	2
Leu	2.4	3
Tyr	1.0	1
Phe	2.3	2
Tml[c]	0.7	1
Lys	1.6	2
His	0.7	1
Arg	2.9	3
Hsr[d]	1.4	2

[a] Mol/mol 5500 M_r peptide
[b] Fragment designation and analysis according to [12]
[c] Trimethyllysine
[d] Determined as homoserine and homoserine lactone

M_r-values of small peptides and their mobility on SDS gels have been reported [10].

The specific residues in the fragment involved in binding to fluphenazine remain to be determined. Phenothiazines may interact with calmodulin in a non-specific manner dependent only on hydrophobic interactions [3,4]. The calcium-induced exposure of hydrophobic regions on calmodulin and other calcium-binding proteins has been reported [16,17]. The 1A–1B fragment may therefore contain such a region. The specificity of this site for phenothiazines and other calmodulin interacting drugs will be the subject of further study.

The inability of the fluphenazine-binding fragment to activate phosphodiesterase may suggest that activation is a property of either the whole calmodulin molecule, or of a region remote from 1A–1B. However, it has been suggested that Met_{109} may be one of the sites of oxidation by *N*-chlorosuccinimide which leads to the loss of biological activity of calmodulin [18]. The conversion to Met_{109} to homoserine by cyanogen bromide while not cleaving the fragment may therefore inactivate an otherwise active region of the molecule. For this reason it is not possible from

these results to preclude this section of the molecule as a site of interaction between calmodulin and phosphodiesterase. However, tryptic peptides encompassing this region of calmodulin have also been reported to have little phosphodiesterase activating ability [19].

Further studies of the fluphenazine-binding fragment should enable us to define the nature of the interaction of phenothiazines at this region of the calmodulin molecule and lead to an improved understanding of the mode of action of these drugs in blocking calmodulin function.

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