Isolation, purification and characterization of intracellular calmodulin like protein (CALP) from *Mycobacterium phlei*

P.V.G.K. Sarma a,*, P. Usha Sarma b, P.S. Murthy c

a Department of Biochemical Technology, Sri Venkateswara College, Dhaula Kuan, New Delhi 110021, India
b Centre For Biochemical Technology, University of Delhi Campus, Mall Road, Delhi 110007, India
c Department of Biochemistry, University College of Medical Sciences, G.T.B. Hospital, New Delhi, India

Received 26 June 1997; revised 17 November 1997; accepted 17 November 1997

Abstract

A monomeric acidic protein of 14,000 Da with an isoelectric point of 4.5 was isolated from *Mycobacterium phlei*, which stained poorly with Coomassie brilliant blue. This protein showed retardation in mobility in SDS-PAGE upon treatment with calcium, similar to eukaryotic calmodulin proteins. Activation of cAMP phosphodiesterase and NAD kinase by this protein was observed. The CD spectral analysis indicated that the CALP has 52% of $\alpha$-conformation. The regular $\alpha$-conformation of the calmodulin like protein was shifted to 46% $\alpha$-helical structure when calcium ions reacted with the protein, however, 42% of the CALP still retained its original $\beta$-conformation. These observations indicated homology of this calcium binding protein with that of eukaryotic calmodulins in few structural and functional properties.

Keywords: *Mycobacterium phlei*; Calmodulin; Calmodulin like protein; SDS-PAGE

1. Introduction

Calmodulin, a ubiquitous unique polypeptide with four calcium binding domains, is present in all higher eukaryotic organisms, and triggers essential metabolic pathways. The $\text{Ca}^{2+}$ calmodulin complex triggered various phosphorylases, phosphodiesterase, NAD kinases, adenylate cyclases, $\text{Ca}^{2+}$, $\text{Mg}^{2+}$ ATPases, etc. [1–13,35]. Besides, it plays a role in the pathogenesis of *E. histolytica* by releasing a secretory collagenase. A calcium binding protein isolated from the ciliate *Tetrahymana pyriformis* with a molecular size of 14,000 Da and isoelectric point of 4.0, activates adenylate cyclase and guanylate cyclase enzymes in a $\text{Ca}^{2+}$ dose dependent manner [5–12]. Few recent reports indicated that prokaryotic organisms too have a calmodulin like protein which resembled eukaryotic calmodulin in calcium binding ability, acidic nature, activation of phosphodiesterases, NAD kinases and cross reacted with the antibodies to eukaryotic calmodulin. Prokaryotic organisms like *Bacillus subtilis*, *Bacillus cereus*, *E. coli*, *Myxococcus xanthus*, *Streptomyces erythreus*, *M. smegmatis* and *Mycobacterium bovis* (BCG) are reported to have calmodulin like protein with molecular masses 15 to 25 kDa and isoelectric points of 4.5–5.0. These molecules stimulated bovine brain...
phosphodiesterase and NAD kinase in a Ca\(^{2+}\) dose dependent manner [15–18,24–27]. In the present study, a calmodulin like protein (CALP) from Mycobacterium \textit{phlei} was isolated and characterized for the first time. Purified protein activated bovine brain cAMP phosphodiesterase and NAD kinase isolated from pea seeds. In view of importance of calmodulin in eukaryotic systems and presence of similar proteins in prokaryotes, a study on molecular characterization of a calcium binding protein that cross reacted with bovine calmodulin antibodies was undertaken.

2. Materials and methods

2.1. Bacterial strains and culturing methods

\textit{M. phlei} (TMC 1548) was used exclusively in this work. The bacteria were cultured in TYE medium, containing 0.2% Tween-20 in 1 l of deionized water [19]. \textit{M. phlei} cells were grown in 1 l conical flasks (Borosil India Ltd.) at 300 rpm and 37°C for overnight in an environmental shaker (Labline Instrument Company, Melrose Park, IL, USA). The cells were pelleted from 15 l of culture media. The cells of \textit{M. phlei} were harvested keeping the cells on ice and the cells were centrifuged at 10,000 rpm at 4°C for 10 min in a Remi refrigerated C-34 centrifuge. The pellet was washed with 50 ml of 50 mM Tris-HCl, pH 7.5 and recentrifuged at 10,000 rpm and 4°C for 10 min. The pellet was then suspended in 15 ml 50 mM Tris-HCl pH 7.5, and these cells were used for the isolation of CALP.

2.2. CALP extraction from \textit{M. phlei}

The \textit{M. phlei} cells were sonicated at a frequency of 50 Hz for 3 min. Completely sonicated cells were then centrifuged at 13,000 rpm at 4°C for 10 min in a Beckman L7-35 Ultracentrifuge. The supernatant was taken and recentrifuged at 35,000 rpm and 40°C for 2 h in a Beckman L7-35 Ultracentrifuge. The supernatant was retained and used as the starting material for the isolation of CALP.

2.3. Identification of CALP by double diffusion method

Anti-bovine brain calmodulin IgG was used in Ouchterlony’s double immunodiffusion method [36].

2.4. Dot ELISA

CALP in the supernatant from the \textit{M. phlei} was monitored by performing dot ELISA [36].

2.5. Purification of CALP

(A) Finely ground ammonium sulfate was added gradually to the extract to a final saturation of 45% (wt/vol) (at 0°C, under constant stirring). The ammonium sulfate precipitate was removed by centrifugation at 15,000 rpm and 40°C for 30 min. The supernatant, thus obtained, was used to concentrate CALP by adding ammonium sulfate gradually to a final saturation of 95% (wt/vol) at 40°C, under constant stirring. The precipitate, thus obtained, was dissolved in 50 mM Tris-HCl, pH 7.5 and dialysed against 50 mM Tris-HCl pH 7.5 and 1 mM CaCl\(_2\), until the ionic concentration of the fraction equated to that of the dialysis buffer. Anti-bovine brain calmodulin reacted with the fraction indicating the presence of CALP. This fraction was further subjected to purification.

(B) CALP was isolated from other contaminants by passing (45–95% (NH\(_4\))\(_2\)SO\(_4\) precipitated fraction) through an anti-bovine brain calmodulin affinity column. This column was prepared by activating 200 mg of Sepharose 4B by cyanogen bromide [34,36].

(C) Purity of CALP on reverse phase HPLC by C-18 column. The peak fraction obtained after immunoaffinity column was further analysed on RP-HPLC using C-18 column 3.9×150 mm; 100 A attached to a Watter 600 s instrument. The solvent used was 0.1% TFA and 80% acetonitrile gradient. The CALP was eluted at a retention time of 4 min in a single peak. The sample was then dialysed against 50 mM Tris-HCl pH 7.5. This protein fraction reacted with anti-bovine brain calmodulin IgG.
Fig. 1. Purification profile of calmodulin like protein (CALP) of \textit{M. phlei}. A: 45–95\% NH\textsubscript{4}(SO\textsubscript{4})\textsubscript{2} fraction was passed through an anti-bovine brain calmodulin immunooaffinity column. Bound CALP was eluted with 50 mM sodium acetate pH 4.5 and 0.15 NaCl. B: Reverse phase HPLC profile of CALP from \textit{M. phlei}, using C-18 column 3.9×150 mm; 100 A. The CALP of \textit{M. phlei} was isolated using 0.1\% trifluor acetic acid (TFA) and 0.1\% TFA and 80\% acetonitrile.

Table 1

Activation of CALP with respect to purification from the \textit{Mycobacterium phlei}

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Purification steps</th>
<th>CALP from \textit{M. phlei}</th>
<th>Minimum conc. of CALP</th>
<th>Bovine heart phosphodiesterase</th>
<th>Pea seed NAD kinase</th>
<th>Reaction with anti-bovine brain calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cytosolic fraction</td>
<td>1 mg/ml</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>45-95% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction</td>
<td>0.1 mg/ml</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Immunoaffinity column</td>
<td>0.01 µg/ml</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Reverse phase HPLC C-18 column</td>
<td>0.01 µg/ml</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
2.6. Polyacrylamide gel electrophoresis
(native PAGE)

The CALP purified on C-18 RP-HPLC was analysed on 17% PAGE gels [20].

2.7. SDS-PAGE

The molecular mass of the purified CALP was analysed on 17% SDS-PAGE [22,23].

2.8. Isoelectric focusing

Isoelectric focusing gels were prepared as 17% polyacrylamide native gel rods [18,21].

2.9. Western blotting

The CALP resolved on 15% SDS-PAGE was electrophoretically transferred to the nitrocellulose paper (NCP) [31] and the nitrocellulose paper was then probed with 1:500 diluted anti-bovine brain calmodulin.

2.10. Calcium mobility shift assay

The purified CALP was run on 12.5% SDS-PAGE in the presence of 10 mM CaCl$_2$ [8,9]. The gel was fixed in 50% methanol and in 10% acetic acid and then stained with 0.1% silver nitrate [21].

### Table 2
Circular dichroism analysis of CALP isolated from *Mycobacterium phlei* [28,30]

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Molar [θ]x$^*$</th>
<th>aCALP</th>
<th>aCALP+10 mM CaCl$_2$</th>
<th>bCALP+20 mM CaCl$_2$</th>
<th>cCALP+30 mM CaCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>[θ]260</td>
<td>-0.709</td>
<td>-0.354</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>[θ]250</td>
<td>-0.887</td>
<td>-0.709</td>
<td>-0.17742</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>[θ]240</td>
<td>-1.064</td>
<td>-1.242</td>
<td>-0.536</td>
<td>-0.709</td>
</tr>
<tr>
<td>4.</td>
<td>[θ]230</td>
<td>-3.548</td>
<td>-1.419</td>
<td>-0.709</td>
<td>-0.709</td>
</tr>
<tr>
<td>5.</td>
<td>[θ]225</td>
<td>-7.806</td>
<td>-1.419</td>
<td>-0.790</td>
<td>-0.354</td>
</tr>
<tr>
<td>6.</td>
<td>[θ]220</td>
<td>-8.871</td>
<td>-1.597</td>
<td>-0.798</td>
<td>-0.354</td>
</tr>
<tr>
<td>7.</td>
<td>[θ]218</td>
<td>-9.758</td>
<td>-1.774</td>
<td>-0.887</td>
<td>-0.374</td>
</tr>
<tr>
<td>8.</td>
<td>[θ]215</td>
<td>-8.161</td>
<td>-1.774</td>
<td>-0.631</td>
<td>-1.064</td>
</tr>
<tr>
<td>9.</td>
<td>[θ]213</td>
<td>-6.387</td>
<td>-0.887</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>[θ]210</td>
<td>-4.435</td>
<td>-0.887</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>[θ]208</td>
<td>-1.774</td>
<td>-0.807</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1, a, b and c denote molar ellipticity measured in deg. cm$^2$ mol $^{-1}$.
2.11. UV absorbance spectrum of CALP

The UV absorption spectrum of purified CALP was scanned on a DU-64 Beckman spectrophotometer against 50 mM Tris-HCl, pH 7.5 against buffer blank in the range of 400 to 200 nm.

2.12. CD spectroscopic studies

The CD spectroscopic studies of purified CALP were carried out on a JASCO-J-500-A spectropolarimeter. Samples were present in 50 mM Tris-HCl pH 7.5. The effect of calcium ions on the CALP was studied in 50 mM Tris-HCl pH 7.5 with varying concentrations of CaCl$_2$ (10 mM CaCl$_2$, 20 mM CaCl$_2$ and 30 mM CaCl$_2$). In all the cases the path length was 0.2 mm with a sensitivity of 1 milli-degree.

2.13. Activation of cAMP phosphodiesterase

The CALP was assayed by stimulating calmodulin deficient cAMP phosphodiesterase [1]. Enzymatic assay was carried out according to Falah et al. [24]. Bovine brain calmodulin 10 ng to 1 mg was added to the reaction mixture and is used as positive control while the reaction mixture without bovine brain calmodulin is used as negative control.

2.14. Activation of NAD kinase

Partially purified NAD kinase was obtained from extracts of pea seeds [4]. Bovine brain calmodulin 10 Table 3

<table>
<thead>
<tr>
<th>S. no.</th>
<th>CALP</th>
<th>CALP+10 mM CaCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fraction of beta</td>
<td>52%</td>
<td>41.7%</td>
</tr>
<tr>
<td>2. Fraction of alpha</td>
<td>20%</td>
<td>45.9%</td>
</tr>
<tr>
<td>3. Fraction of random coil</td>
<td>28%</td>
<td>12.3%</td>
</tr>
</tbody>
</table>

Fig. 3: A: 17% isoelectric focusing gel of CALP isolated from M. phlei. 15 µg of protein was used in the experiment. B: 17% SDS-polyacrylamide gel electrophoresis of CALP isolated from M. phlei. Lane 1, CALP of M. phlei purified by passing through the RP-HPLC (C-18) column; lane 2, CALP of M. phlei isolated from anti-bovine brain immunoaffinity column; and lane 3, standard molecular mass markers (65 kDa bovine serum albumin, 45 kDa egg albumin, 30 kDa carbonic anhydrase, 21 kDa chymotrypsin, 14 kDa lysozyme and 12 kDa cytochrome c). C: Calcium mobility shift assay: 12.5% SDS-polyacrylamide gel electrophoresis of CALP treated with CaCl$_2$. Lanes 1 and 2, CALP without CaCl$_2$ treatment; lane 3, 45–95% (NH$_4$)$_2$SO$_4$ precipitated fraction; lanes 4 and 5, CALP treated with 10 mM CaCl$_2$. 

ng to 1 μg was added to the NAD kinase reaction mixture and is used as positive control while the reaction mixture without calmodulin or CALP is used as negative control [18].

3. Results

The cytosolic proteins of *M. phlei* reacted with anti-bovine brain calmodulin antibodies in DID and in dot ELISA. This suggested the presence of CALP in *M. phlei*. A major fraction containing a low molecular mass CALP protein was concentrated between 45–95% (NH₄)₂SO₄. This fraction activated phosphodiesterase and NAD kinase (Table 1). Table 1 indicates the purification procedure with respect to activation of phosphodiesterase and NAD kinase and reaction with the antibodies raised against bovine brain calmodulin. Fig. 1 indicates the purity of CALP by immunoaffinity and C-18 RP-HPLC columns. The purified protein gave a precipitin band in DID, and single band on Western blot with antibodies to bovine brain calmodulin (Fig. 2). SDS-PAGE of the purified protein showed single band with a molecular mass of 14000 Da and an isoelectric point of 4.5. The molecular mass of calcium treated CALP showed a decrease in the mobility on SDS-PAGE of 4.5. The molecular mass of calcium treated CALP was found to be 14000 Da and pI value 4.5 similar to eu karyotic calmodulins and CALP of few prokaryotes [14–18,24–32,33]. The UV spectrum of CALP from *M. phlei* showed maxima at 238 nm and 205 nm, indicating absence of tryptophan and tyrosine residues, respectively. This observation corroborated with the findings of Fry et al. [18] for *B. subtilis*. The CD spectrum of CALP showed a high amount of β-pleated sheet structure, similar to CALP of *M. xanthus* and *S. erythreus* [25–27]. However, as Ca²⁺ ions bound to the CALP, a distinctive conformational change to α-helical structure was observed though a significant portion of the CALP still retained β-pleated sheet structure. This arrangement, of a α-helical and β-loop together was characteristic of all known eukaryotic calcium modulating proteins [25–27]. This was also the characteristic feature of calcium binding protein isolated from *S. erythreus* [25]. This fact further emphasized that when Ca²⁺ ions bind, the protein undergoes a major conformational change to trigger a cascade Ca²⁺ dependent reaction. However, the CALP isolated from *B. subtilis*, *B. cereus*, *E. coli*, *M. smegmatis* and *M. bovis* did activate phosphodiesterase and NAD kinase in a calcium dependent manner [17,18,24].

In the current study, a cytosolic protein from *M. phlei* with calmodulin like properties was observed and the protein was purified to homogeneity. Biochemical and immunochemical characterization of the purified protein showed a molecular mass of 14000 Da and a pI of 4.5, and it reacted with ant bovine brain calmodulin antibodies. The purified protein bound to calcium ions, activated phospho-

4. Discussion

Calmodulin like protein from *M. phlei* was purified and characterized. The biochemical and biophysical properties of the molecule differed from the eu karyotic calmodulins. CALP isolated from *M. phlei* reacted with anti-bovine brain calmodulin in double immunodiffusion test and on Western blot. The purified protein (obtained from immunoaffinity column and on further resolution on C-18 RP-HPLC column) was able to activate cAMP phosphodiesterase and NAD kinase from pea seeds (Table 1) similar to eu karyotic calmodulin and the CALP from *B. subtilis*, *B. cereus*, *E. coli*, *M. smegmatis* and *M. bovis* (BCG). The molecular mass of CALP from *M. phlei* was observed to be 14000 Da and pI value 4.5 similar to eu karyotic calmodulins and CALP of few prokaryotes [14–18,24–32,33]. The UV spectrum of CALP from *M. phlei* showed maxima at 238 nm and 205 nm, indicating absence of tryptophan and tyrosine residues, respectively. This observation corroborated with the findings of Fry et al. [18] for *B. subtilis*. The CD spectrum of CALP showed a high amount of β-pleated sheet structure, similar to CALP of *M. xanthus* and *S. erythreus* [25–27]. However, as Ca²⁺ ions bound to the CALP, a distinctive conformational change to α-helical structure was observed though a significant portion of the CALP still retained β-pleated sheet structure. This arrangement, of a α-helical and β-loop together was characteristic of all known eukaryotic calcium modulating proteins [25–27]. This was also the characteristic feature of calcium binding protein isolated from *S. erythreus* [25]. This fact further emphasized that when Ca²⁺ ions bind, the protein undergoes a major conformational change to trigger a cascade Ca²⁺ dependent reaction. However, the CALP isolated from *B. subtilis*, *B. cereus*, *E. coli*, *M. smegmatis* and *M. bovis* did activate phosphodiesterase and NAD kinase in a calcium dependent manner [17,18,24].

In the current study, a cytosolic protein from *M. phlei* with calmodulin like properties was observed and the protein was purified to homogeneity. Biochemical and immunochemical characterization of the purified protein showed a molecular mass of 14000 Da and a pI of 4.5, and it reacted with anti bovine brain calmodulin antibodies. The purified protein bound to calcium ions, activated phospho-
diesterases and NAD kinases similar to eukaryotic calmodulins. The major portion of the CALP molecule was in the β-pleated structure. However, when CALP binds to Ca ions it undergoes a distinctive conformational change where more α-helical structures were observed. In view of the immuno-cross-reactivity with anti-bovine brain calmodulin and similarities in biochemical properties with eukaryotic and prokaryotic calmodulin, the CALP of *M. phlei* is anticipated to have a similar physiological and biochemical role in *M. phlei*. Further work on molecular characterization of this protein and its role is in progress.

References


