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Isolation and Characterization of a Calmodulin-Like Protein from *Halobacterium salinarium*

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The first evidence for a calmodulin-like protein in an archaeon, *Halobacterium salinarium*, is reported here. The calmodulin-like protein, with a molecular mass of 24 kDa and an estimated pI of 4.8, stimulated cyclic nucleotide phosphodiesterase in a calcium-dependent manner. This stimulation could be suppressed by calmodulin inhibitors. The Ca^{2+}-binding ability was verified by ^{45}Ca autoradiography.

Calmodulin, a small, acidic, heat-stable, Ca^{2+}-binding protein, has been found in many and is inferred to be present in nearly all eucaryotic cells. Calmodulin was first reported as a protein activator of bovine brain phosphodiesterase (4) and is now known to activate several enzymes of physiological importance (1, 12).

The presence of calmodulin-like proteins (CaLP) in procaryotes has been reported by several authors. CaLP and calcium-mediated regulation in bacteria have been critically reviewed by Onek and Smith (14); the authors present a complex picture in which no single protein is fully identified as a calmodulin. The analogy drawn between calmodulin and the procaryotic proteins (CaLP) has relied mainly on characteristic properties of calmodulin, such as Ca^{2+} binding and enzyme stimulation. In matching these criteria, we present evidence for a calmodulin-like protein in the archaeon *Halobacterium salinarium* (previously known as *Halobacterium halobium*).

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H. salinarium mutant strain Flx.-03 l.c., of low carotenoid content and deficient in bacteriorhodopsin and halorhodopsin (18), was used throughout this work. Cells were exclusively grown at 40°C in growth medium (21) under semianaerobic conditions described earlier (20). The cells were centrifuged from the growth medium, 12 days after inoculation, at 12,000 × *g* and 4°C for 15 min, washed with 4.28 M NaCl–81 mM $MgSO_4$–10 mM KCl, and centrifuged again. The cell pellet was frozen and stored at −70°C. The thawed cell pellet was mortared and sonified (for 5 min, on a 20 to 50% duty cycle, and with a microtip 6 on a B15 sonifer cell disruptor; Branson, Heusenstamm, Germany) with alumina and breakage buffer containing 2.2 M $(NH_4)_2SO_4$, 40 mM $MgSO_4$, and 10 mM Tris-HCl (pH 7.5).

The crude extract was digested (DNase I) at room temperature for 1 h and centrifuged at 35,000 × *g* and 4°C for 30 min. The supernatant was stored. Soluble proteins still enclosed in the pellet were eluted and centrifuged in breakage buffer three times. The supernatant fractions were pooled, heated to 75°C in a microwave oven, and then cooled on ice. The heat-denatured material was removed by centrifugation at 17,000 × *g* and 4°C for 40 min and then by ultracentrifugation at 150,000 × *g* and 4°C for 3 h.

For isoelectric precipitation the supernatant was adjusted to pH 4 and stirred at 4°C for 1 h. All proteins with isoelectric points other than around pH 4 stayed soluble. After centrifugation at 17,000 × *g* and 4°C for 40 min, the pellet was resuspended in a solution of 100 mM NaCl, 5 mM $CaCl_2$, 1 mM dithiothreitol (DTT), and 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.5) and stirred on ice for 1 h while the pH was constantly brought back to pH 7.5. Unsoluble material was removed by centrifugation (see above).

The resuspended isoelectric precipitate was applied to a Q-Sepharose fast-flow column (HR 5/5; Pharmacia, Freiburg, Germany) at a flow rate of 0.5 ml/min and at 4°C. After the sample was loaded, the column was washed until the A_{280} reached the lowest stable background value. The proteins were eluted with a gradient of NaCl (0 to 1 M) in the MOPS buffer (pH 7.5). The peak, eluted at about 200 mM NaCl, stimulated the phosphodiesterase in the enzyme assay.

The phosphodiesterase-stimulating peak was diluted at a ratio of 1:3 with 1 mM DTT–50 mM MOPS (pH 7.5), and $CaCl_2$ was added to 5 mM. The Ca^{2+}-enriched protein solution was loaded on a 2-chloro-10-(-3-aminopropyl)-phenothiazin (CAPP) affinity column (8) (XK 16/20 or HR 10/10; Pharmacia) at 4°C with a flow rate of 115 μl/min; the calmodulin inhibitor CAPP was coupled to CNBr-activated Sepharose 4B (Pharmacia; coupling process according to the instructions of the manufacturer). The calcium-bound CaLP was able to interact with CAPP. The column was washed with equilibration buffer containing 100 mM NaCl, 5 mM $CaCl_2$, 1 mM DTT, and 50 mM MOPS (pH 7.5) until the A_{280} reached the lowest stable background value. The CaLP could be eluted by chelation of calcium ions with 10 mM ethylene glycol tetraacetic acid (EGTA) in the buffer. The eluted peak stimulated the phosphodiesterase in the enzyme assay and appeared as a single protein band of 24 kDa by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (data not shown).

The silver-stained (3) two-dimensional electrophoresis (13) nonequilibrium pH gradient electrophoresis (NEPHGE)-SDS gel showed the CaLP to be an acidic protein of about 24 kDa (Fig. 1); an isoelectric point of 4.8 was estimated by isoelectric focusing gels prepared as ultrathin gels in the range of pH 3 to 10 (5).

The molecular mass of 24 kDa was calculated from the data obtained by SDS-PAGE and gel filtration (column XK 26/70 [Pharmacia] with a gel bed height of 63 cm of Sephadex G-150 superfine [Sigma, Deisenhofen, Germany] at room temperature and a flow rate of 0.18 ml/min).

The CaLP from the archaeon *H. salinarium* shows similari-

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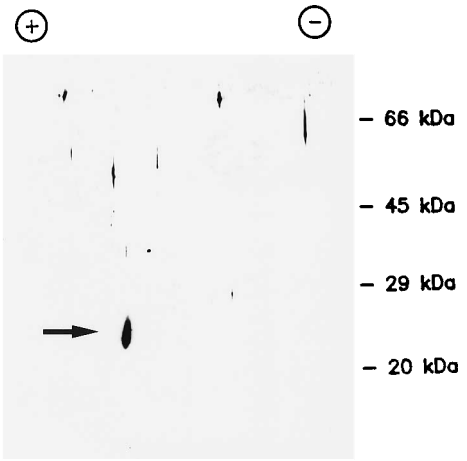

FIG. 1. Two-dimensional electrophoresis of the peak of the affinity chromatogram. The NEPHGE-SDS gel shows, in the first dimension (horizontal), separation by isoelectric focusing. The more acidic proteins stay on the side of the anode (⊕), while the more basic ones move to the cathode (⊖). The second dimension (vertical) is separation by molecular mass. The CaLP appeared as a single protein band of 24 kDa in SDS-PAGE. CaLP is given here by a spot on the more acidic side (arrow).

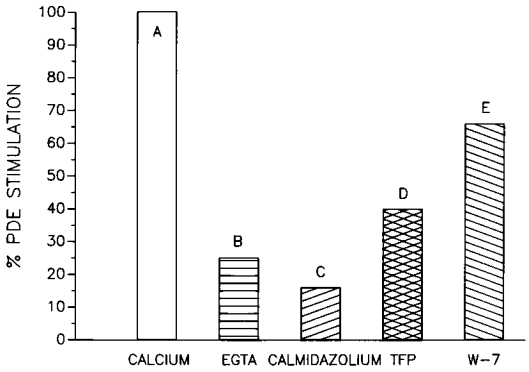

FIG. 2. Influence of calmodulin inhibitors and the calcium chelator EGTA on the stimulation of cyclic nucleotide phosphodiesterase (PDE) by CaLP from *H. salinarium*. In the presence of 20 mM $CaCl_2$ the stimulation is set to 100% (A). In the presence of 20 mM EGTA the stimulation is reduced to 25% (B), while the calmodulin inhibitor calmidazolium at a concentration of 1.2 μM decreased the stimulation to 16% (C), 30 μM trifluoperazine (TFP) decreased the stimulation to 40% (D), and 30 μM W-7 decreased the stimulation to 66% (E).

ties in molecular mass to those of the respective proteins of *Bacillus subtilis* and *Bacillus cereus* (25 and 24 kDa, respectively [6, 17]) but appears to be larger than mammalian calmodulin and plant calmodulins (17.6 and 18.6 kDa, respectively [22, 23]). The isoelectric point of CaLP from *H. salinarium* falls in the same range as that of CaLP from *B. subtilis* (4.9 to 5.0 [6]) but, again, differs from that of eucaryotic calmodulin (3.9 [11]).

The ability of *H. salinarium* CaLP preparations to stimulate bovine heart phosphodiesterase was tested by an enzyme assay based on the protocol of Jacobshagen et al. (7). The reaction medium contained, in a volume of 0.84 ml, 4 mM $MgSO_4$, 0.2 mM $CaCl_2$, 100 mM imidazole, 2 mM cyclic AMP, 5 U of alkaline phosphatase, 0.5 mU of calmodulin-deficient cyclic nucleotide phosphodiesterase from bovine heart at pH 7.5, and various amounts of porcine brain calmodulin or CaLP from *H. salinarium*. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by cooling on ice. After adding 0.16 ml of a solution of 30 mM ascorbic acid, 1.2 M sulfuric acid, 1.6% (wt/vol) SDS, and 0.6% (wt/vol) ammonium molybdate, the mixture was incubated at 60°C for 30 min and cooled on ice. The A_{280} was determined.

The concentration dependence of the stimulation of bovine heart phosphodiesterase with crude extract of *H. salinarium* after heat treatment was found to be comparable to that of the one with porcine brain calmodulin, although the absolute amounts (estimated for CaLP) differ (data not shown).

A requirement of Ca^{2+} by the CaLP, to stimulate the phosphodiesterase, was verified by the use of the calcium chelator EGTA, which repressed the enzymatic stimulation by 75% (Fig. 2, columns A and B).

The calmodulin inhibitors trifluoperazine (Röhm Pharma, Darmstadt, Germany) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and compound R24571 (calmidazolium) (Sigma) were tested in the enzyme assay. W-7, at a concentration of 30 μM, reduced the stimulation with CaLP by 34%, while 30 μM trifluoperazine had an effect of 60% reduction. The potent specific calmodulin inhibitor calmidazolium, at a concentration of 1.2 μM, decreased the stimulation with CaLP by 84% (Fig. 2, columns E, D, and C, respectively).

It should be mentioned that for partially purified CaLP, the effect of the calmodulin inhibitors was less distinct than shown in Fig. 2, while purified CaLP fractions were sometimes inhibited up to 100%. This inhibition could be a function of variable protein concentrations or due to removal of a component which interferes in the process (15).

The calcium-binding ability of CaLP, indicated by the inhibitory effect of the calcium chelator EGTA on stimulation of the phosphodiesterase, was verified directly by ^{45}Ca autoradiography (10). After SDS-PAGE, protein was electrophoretically transferred (19) from the gel to an Immobilon polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) at a constant current of 200 mA at room temperature for 2 h. After electrophoretic transfer, the membrane was incubated for 30 min in 1 to 2% glutaraldehyde, washed twice for 15 min with 40 mM NaCl–5 mM $MgCl_2$–10 mM imidazole (pH 6.8), incubated for 10 min in the buffer containing 0.5 μM ^{45}Ca (1 mCi/liter),

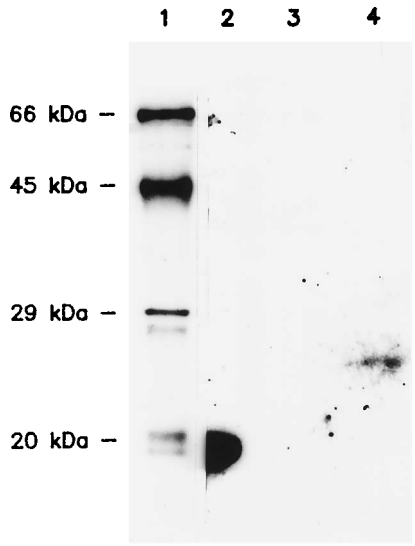

FIG. 3. Autoradiogram showing ^{45}Ca binding to the CaLP of *H. salinarium*. Lane 1, molecular mass markers of the SDS-polyacrylamide gel prior to the electrophoretic transfer; lane 2, positive control, porcine brain calmodulin; lane 3, negative control, BSA; lane 4, CaLP from *H. salinarium*.

and washed twice for 2.5 min (each wash) in 50% (vol/vol) ethanol at room temperature. The air-dried membrane was exposed to X-ray film (Hyperfilm-MP; Amersham, Braunschweig, Germany) at −70°C for 3 to 21 days. The autoradiogram demonstrated the calcium-binding ability of CaLP in reference to those of bovine serum albumin (BSA) as the negative control and porcine brain calmodulin as the positive control (Fig. 3, lanes 4, 3, and 2, respectively).

Because of the lack of a reliable quantitative assay for the CaLP, especially in crude extracts of *H. salinarium* cells prior to heat treatment, no precise quantitative data are available. The approximate concentration of the CaLP was calculated as 0.1 μM/kg of cell pellet.

The identification of putative bacterial calmodulins indicates calcium-mediated regulation in procaryotic organisms (2, 6, 14). Conclusive evidence concerning the function of a Ca^{2+}-binding CaLP in *H. salinarium* is not yet available. Schimz and Hildebrand (16) describe the influence of Ca^{2+} and cyclic GMP on the sensory behavior of *H. salinarium*. They suggest that Ca^{2+} and cyclic GMP might be antagonistic components of an oscillator, which, from their theory (recently challenged by Krohs [9]), generates the autonomous rhythm of flagellar reversals. Furthermore, Ca^{2+} is thought to act on the methylation system which determines the lifetime of sensory signals at an integration link prior to the oscillator and thereby allows adaptation (16).

The finding of CaLP in *H. salinarium*, as reported here, substantiates the concept of Ca^{2+}-mediated regulation in procaryotic cells and extends the family of CaLP to members of the domain *Archaea*.

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