Review Article

Calmodulin and calcium mediated regulation in prokaryotes

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Introduction

In the eukaryotic cell the calcium ion, Ca^{2+} , is known to act as an effector of stimulus–response coupling in the regulation of diverse cellular functions through a mechanism which is triggered by a varied assortment of hormonal and environmental stimuli. In response to the stimulus the cytosolic concentration of free Ca^{2+} ($[\text{Ca}^{2+}]_i$) is elevated either via an increased influx of extracellular Ca^{2+} through calcium channels in the plasma membrane or by the release of sequestered Ca^{2+} from intracellular inclusions such as the endoplasmic and sarcoplasmic reticula. The latter response may be triggered by other effectors, such as inositol triphosphate, which are synthesized/released in response to the evoking stimulus (Berridge & Irving, 1989). The increase in $[\text{Ca}^{2+}]_i$ displaces the equilibrium of a family of calcium binding proteins which, when bound to Ca^{2+} possess the capability of increasing the rate constant of a variety of enzyme catalysed reactions. Finally, the $[\text{Ca}^{2+}]_i$ declines to the resting, steady state concentration by the action of Ca^{2+} pumps which return the Ca^{2+} to the intracellular inclusion or the external medium (Rasmussen, 1989).

The concept of a family of calcium binding proteins that could transduce a Ca^{2+} mediated signal into a physiological response originated with the work of S. Ebashi (Ebashi & Kodama, 1965), who demonstrated that troponin C, a Ca^{2+} binding subunit of the troponin complex, conferred Ca^{2+} sensitivity to the contractile structure of skeletal muscle cells. Subsequently, an activator of cyclic nucleotide phosphodiesterase (PDE), an enzyme which is involved in the regulation of cAMP concentration, was described by Cheung (1970). Further work (Kakiuchi & Yamazaki, 1970) showed that a particular form of PDE was stimulated by calcium in the presence of a protein factor. The factor was shown to

Table 1. *Examples of Ca^{2+} /calmodulin dependent cellular processes in eukaryotes*

See Klee & Vanaman (1982), Marme (1988) and Allan & Hepler (1989).

Cell motility	Cell elongation
Cyclic nucleotide metabolism	Cell division
Ca^{2+} transport systems	Protoplasmic streaming
Protein phosphorylation	Enzymic secretion
Immune action	Neurotransmitter release
Turgor regulation	Microtubule disassembly
RNA transcription	Enzymic activities

bind calcium (Teo & Wang, 1973) and recognition of its relationship to troponin followed quickly (e.g. Watterson *et al.*, 1976). With the discoveries of the ability of these proteins to stimulate bovine brain adenylyl cyclase (Bostrom *et al.*, 1975), and of the Ca^{2+} dependent ATPase of mammalian erythrocytes (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977) and protein kinases (e.g. Schulman & Greengard, 1978), the wide ranging regulatory function of this Ca^{2+} binding protein first became apparent. Eventually renamed 'calmodulin', (Cheung *et al.*, 1978), the protein rapidly became established as a 'ubiquitous' (Klee & Vanaman, 1982; Klee, 1988; Means *et al.*, 1982) component of, initially, animal cells, and later, all eukaryotic cells. Calmodulin is currently regarded as a central component in a complex regulatory mechanism which interacts with other regulatory effectors (e.g. cAMP) and thus imposes control upon many of the essential metabolic and physiological functions of the cell (Klee & Vanaman, 1982; Klee, 1988; Means *et al.*, 1982) (Table 1).

Calmodulin is a heat stable, monomeric, globular protein and has, depending upon the eukaryotic source of the protein, a molecular mass ranging from 15–22 kDa (Burgess & Kretsinger, 1981; Fry *et al.*, 1986). The monomer is encoded by a single gene which is considered to be highly conserved. Among the vertebrates it consists

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Abbreviation: PDE, cyclic nucleotide phosphodiesterase.

of about 148 amino acid residues; a high proportion of these are acidic (27 glutamate and 23 aspartate residues), resulting in an isoelectric point in the range 3.9–4.3. Regardless of source, those calmodulins analysed so far lack tryptophan and, with a few exceptions among higher plants (peanut, spinach, wheat and zucchini), also lack cysteine (Marmé, 1988). Eukaryotic calmodulins also possess an unusually high ratio of phenylalanine to tyrosine residues which results in an atypical UV absorption spectrum. Instead of the single maximum at 278–280 nm, characteristic of most proteins, calmodulin generates a series of maxima at 253, 259, 265, 268 and 276 nm (Klee & Vanaman, 1982). Another unusual and distinct characteristic of calmodulin is observed on SDS-PAGE. Despite the denatured condition of the protein in this procedure, it displays different mobilities depending upon whether Ca^{2+} is present (Watterson *et al.*, 1980; Vanaman, 1983; Garrigos *et al.*, 1991; Jablonsky *et al.*, 1991). Even when denatured, calmodulin is able to bind Ca^{2+} (Maruyama *et al.*, 1984) and appears able to adopt secondary and tertiary structures that result in an apparent 35% decrease in the molecular mass of the protein (Garrigos *et al.*, 1991). This characteristic provides an excellent additional criterion for the identification of calmodulin.

The concept of evolutionary conservation of calmodulin was originally drawn from studies of the protein from vertebrate sources (e.g. Klee & Vanaman, 1982), but has suffered little from the additional plant and lower eukaryote sequences now available. For instance, plant and vertebrate calmodulins are distinguished merely by the presence of one or two tyrosine residues respectively (Marmé, 1988). While the degree of homology between diverse eukaryotic sources is high, there are, however, immunological differences. Thus polyclonal antibodies raised against spinach calmodulin show little if any cross reaction with vertebrate or protozoan calmodulins, but react well against other plant calmodulins (Schleicher *et al.*, 1983; Watterson *et al.*, 1984; Jablonsky, *et al.*, 1991). This may be an aspect of the conservation of calmodulin. Most of the epitopes presented to the immune system of an animal by a foreign calmodulin will be identical to those of the animal's own calmodulin and therefore will be considered to be 'self' antigens. The tolerance of animals to self antigens requires that the means to respond to them will be absent. Consequently, only those epitopes which differ from those of the animal's own calmodulin would promote antibody production. Such antibodies will reflect the differences, rather than the similarities, between animal and other calmodulins.

The evolutionary conservation of calmodulin extends to the repetitive internal structure which, like that of the other 'EF hand' group of proteins (see Kretsinger &

Nockolds, 1973, for the definition of the 'EF hand' concept) to which calmodulin belongs, is divided into four structurally and functionally similar domains (viz. Klee & Vanaman, 1982). Along the primary structure of calmodulin there exists a higher degree of sequence homology between the alternate domains than between adjacent domains (i.e. I & III and II & IV). This repetitive structure has prompted the hypothesis that the four domains arose from two duplication events (Goodman, 1981), which must have been separated by sufficient time to allow divergence between the two halves of the first duplication. Both duplication events must have preceded the common progenitor of all present day EF hand proteins. Each domain incorporates a high affinity Ca^{2+} binding site (Klee & Vanaman, 1982; Means *et al.*, 1982). Calmodulin also binds a variety of compounds, such as phenothiazines (e.g. trifluoperazine) (Weiss *et al.*, 1985) used in the treatment of psychosis, and naphthalene sulphonamide derivatives (e.g. W-7) (Hidaka *et al.*, 1979). These compounds inhibit Ca^{2+} /calmodulin dependent processes (Hartshorne, 1985) and are one means by which such processes may be identified, though caution is required in the interpretation of experiments because the compounds are not specific in their action and their effects vary in different tissues (Roufogalis, 1981). The compounds also enable the purification of calmodulin by affinity chromatography (Charbonneau & Cormier, 1979).

The ability of calmodulin to stimulate enzyme activity appears to reside in a hydrophobic region of the protein that is exposed in the conformational change which occurs on the binding of Ca^{2+} (LaPorte *et al.*, 1980). The exposure of this hydrophobic region permits the purification of calmodulin by hydrophobic interaction chromatography on resins such as phenyl-Sepharose (Gopalakrishna & Anderson, 1982). Calmodulin binding is thought to promote changes in the conformation of the target protein which influence, positively or negatively, its enzymic function (LaPorte *et al.*, 1980). Calmodulin interacts with a large number of different enzymes, which implies that the interaction must be relatively consistent. The conservation of calmodulin structure together with this consistency probably accounts for the ability of the protein isolated from one source to activate enzymes extracted from another species, an ability that extends across Kingdom boundaries. Consequently, it is feasible to assay a plant calmodulin by its ability to activate bovine brain PDE (Anderson & Cormier, 1978). However, calmodulin interacts only with distinct target proteins, having no effect on others. This interaction with a range of target proteins may be responsible for the evolutionary conservation. Some examples of known Ca^{2+} /calmodulin dependent enzymes are shown in Table 2.

Table 2. Some Ca^{2+} /calmodulin dependent enzymes in eukaryotes

See Klee & Vanaman (1982), Means *et al.* (1982), Van Eldik & Waterson (1985) and Allan & Hepler (1989).

Cyclic nucleotide phosphodiesterase	O-Methyltransferase
Adenylate cyclase	Phosphoprotein phosphatase
Phosphofructokinase	Phospholipase A_2
Guanylate cyclase	Tryptophan 5' monooxygenase
cGMP protein kinase	Succinate dehydrogenase
Myosin light chain kinase	N-Methyltransferase
($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase	NAD kinase
Phosphorylase kinase	Superoxide dismutase
Glycogen synthase kinase	Plant lipooxygenase

As calmodulin oriented research diverged to include many different eukaryotic species, certain properties of calmodulin were adopted as criteria for the identification of a novel protein as calmodulin. Given the conserved nature of calmodulin throughout the eukaryotes, the criteria are readily justified. However, the use of such criteria in the identification of a protein from a prokaryotic source is less well founded and must depend upon the assumption that conservation extends to such putative prokaryotic calmodulins.

Prokaryotic calmodulin

Bacterial calmodulins

The first published claim to the discovery of a prokaryotic calmodulin was probably that of Iwasa *et al.* (1981), who described the presence of a heat stable factor in *Escherichia coli* which exhibited a Ca^{2+} dependent activation of PDE, ATPase and myosin light chain kinase. This claim contradicted an earlier report by Grand *et al.* (1980), who found no evidence for calmodulin in *E. coli*. Such conflicting results are likely to have reinforced the prevailing view of the time that calmodulin did not exist in bacteria (Klee & Vanaman, 1982; Campbell, 1983). Harmon *et al.* (1985) reported the presence in extracts of *E. coli* of at least three heat stable proteins (mol. mass 33, 47 and 60 kDa), which preferentially bound $^{45}\text{Ca}^{2+}$ on suspension in buffers containing micromolar concentrations of Ca^{2+} and physiological salt concentrations. Crude fractions containing the proteins were not able to activate NAD kinase, but the total crude extract contained an antigen to a commercially prepared anti-calmodulin antibody. Harmon *et al.* (1985) also reported, in brief, a Southern blot analysis in which a probe derived from an eel calmodulin gene (Lagace *et al.*, 1983) detected complementary sequences in restricted genomic DNA from a variety of prokaryotes

(*E. coli*, *Bacillus cereus*, *Myxococcus xanthus*, *Pseudomonas putida*, *Anabaena* species, *Methanosarcina barkeri*, *Desulfovibrio vulgaris*, *Acinetobacter calcoaceticus*). However, results were not shown and the exact nature of the experiments were not given (e.g. stringency conditions). Such complementarity should have led to the isolation of putative prokaryotic calmodulin genes. In a similar experiment, probes derived from a chicken calmodulin gene (Putkey *et al.*, 1983) detected complementarity to three restriction fragments (7, 5 and 3 kb) of the cyanobacterium *Nostoc* PCC 6720 (a species related to *Anabaena*) genomic DNA at low stringency (50 °C, $1 \times \text{SSC}$). However, similar complementarity was found to the vector DNA (pBR322) which, as a contaminant of the probe preparation, would account for the apparent hybridization of the probe (Temple, 1989).

One of the species used by Harmon *et al.* (1985), *Myxococcus xanthus*, is known to produce a developmentally specific protein, called protein S. The assembly of protein S in *M. xanthus* spores requires Ca^{2+} . Amino acid sequencing of protein S revealed that it contains four domains which share homology with those of calmodulin (Inouye *et al.*, 1983). Domains I and III contain the amino acid sequence

Glu-Asp-Asn-Asn-Thr-Ile-Ser-Ser-Val-Lys

which compares to the Ca^{2+} binding sequence of bovine brain calmodulin (the putative Ca^{2+} -binding residues are underlined).

Asp-Gly-Asn-Gly-Thr-Ile-Thr-Lys

Protein S is similar to calmodulin with respect to size, heat stability, pI, the lack of cysteine residues and the hydrophobic amino acid content. However, the two proteins differ in that protein S has a higher proline content and has a lower affinity for Ca^{2+} than calmodulin. It is not known whether protein S is able to activate Ca^{2+} /calmodulin dependent enzymes.

Saccharopolyspora erythraea (formerly known as *Streptomyces erythraeus*) contains a protein (mol. mass 20.1 kDa) that was serendipitously discovered and isolated by Leadlay *et al.* (1984), and was found to possess characteristics in common with calmodulin. When subjected to SDS-PAGE, the protein exhibited the Ca^{2+} dependent mobility shift and direct binding studies using $^{45}\text{Ca}^{2+}$ demonstrated that it has a high affinity for Ca^{2+} . The primary sequence of 177 residues of this protein is somewhat larger than that of calmodulin and although it possesses a high percentage of acidic residues, the overall amino acid composition is distinctly different (Swan *et al.* 1989). The *S. erythraea* protein does not activate PDE, nor does it show any reaction with antibodies raised against bovine calmodulin. However, the protein contains regions homologous to calmodulin that corre-

Table 3. Amino acid composition of *Bacillus calcium binding proteins* and bovine brain calmodulin

Amino acid	Residues mol ⁻¹ in:		
	<i>B. cereus</i> *	<i>B. subtilis</i> †	Bovine brain‡
Asp + Asn	24	11	23
Thr	5	11	12
Ser	10	63	4
Glu + Gln	43	33	27
Pro	4	8	2
Gly	22	48	11
Ala	23	19	11
Val	18	6	7
Met	1	1	9
Ile	6	5	8
Leu	25	5	9
Tyr	1	8	2
Phe	10	4	8
His	6	11	1
Lys	15	3	7
Arg	9	2	6
Trimethyllysine	n	n	1
Trp	n	n	0
Cys	0	0	0
Total	222	238	148

* Shyu & Foegeding (1991). † Fry *et al.* (1991). ‡ Watterson *et al.* (1980).

n, Not reported.

spond to potential Ca²⁺ binding sites in both proteins (Leadlay *et al.* 1984; Swan *et al.*, 1987; 1989).

The first example of calmodulin-like activity in a Gram-positive bacterium, *Bacillus subtilis*, was reported by Fry *et al.* (1986). A heat stable factor present in the soluble fraction of cell free extracts derived from sporulating cultures was found to stimulate PDE in the presence of Ca²⁺ and the stimulation was inhibited by trifluoperazine. The factor bound to phenothiazine-agarose in the presence of Ca²⁺ and was eluted in buffers containing the Ca²⁺ chelating moiety EGTA. In addition, the factor displaced authentic bovine calmodulin from its antibody in radioimmunoassays. On purification (which was based on procedures employed in the isolation of authentic calmodulins), the factor was shown to be a protein (mol. mass 23–25 kDa) with all the characteristics of the crude preparation and the additional ability to activate NAD kinase from pea shoot tissue. The *B. subtilis* protein has a pI of 4.9–5.0; this figure is consistent with the reported acidic amino acid content, though higher than that of eukaryotic calmodulins. It contains a preponderance of glycine and serine in quantities several times greater than the respective amounts of the two residues in bovine brain calmodulin. The two proteins differ significantly both in the general composition and the amount of the amino acid residues (Table 3).

Other characteristics of the *B. subtilis* protein that are reported to differ from those of eukaryotic calmodulin include: (1) complete, irreversible loss of the ability to stimulate PDE following removal of Ca²⁺ either by chelation or dialysis; (2) following labelling with tetramethylrhodamine isothiocyanate (TMRITC), the protein shows accelerated mobility on SDS gel, loss of antigenicity towards anti-bovine-calmodulin antibodies and loss of the ability to stimulate PDE; (3) the protein has an absorbance maximum at 290 nm compared with 277 nm region for bovine calmodulin. It also lacks the fine structure in the 250–270 nm region, which is ascribed to the absorbance of phenylalanine in eukaryotic calmodulins (Fry *et al.*, 1991).

A similar heat stable, calcium binding protein has also been purified from dormant spores of *Bacillus cereus* by Shyu & Foegeding (1991). The molecular mass of the protein is estimated to be 24 kDa based on amino acid content and SDS-PAGE. It binds ⁴⁵Ca²⁺, as determined by the method of Maruyama *et al.* (1984), and is retained on phenyl-Sepharose matrices in a calcium dependent fashion. It is not known whether the *B. cereus* protein possesses the Ca²⁺ dependent ability to activate phosphodiesterase or NAD kinase. The amino acid composition of the protein has been determined (Table 3) and is distinctly different from that of the bovine brain calmodulin.

Cyanobacterial calmodulins

Kerson *et al.* (1984) were probably the first to report the existence of a calmodulin-like protein in a cyanobacterium. It was known that low concentrations of Ca²⁺ in the growth medium stimulate the uptake of inorganic phosphate and its deposition as polyphosphate granules. Kerson *et al.* (1984) reported that the stimulation observed in cultures of *Oscillatoria limnetica* was inhibited by fluphenazine, an anti-psychosis drug that is known to inhibit Ca²⁺/calmodulin dependent processes in eukaryotes, and that an antigen to a commercially prepared anti-calmodulin antibody was present in cell free extracts of *O. limnetica*. This brief communication was followed by the more substantial report of Pettersson & Bergman (1989), which described the detection of a calmodulin-like activity in three strains of the genus *Anabaena*. Boiled crude extracts of these strains were able to stimulate an NAD kinase isolated from cucumber in a Ca²⁺ dependent reaction that was inhibited by EGTA and the calmodulin inhibitor chlorpromazine. Western blot analysis using a polyclonal antibody raised against spinach calmodulin (Van Eldik & Wolchok, 1984) detected a protein of about 17 kDa. An important aspect of the report was the localization of the protein by electron microscopy to both the vegetative cells and

heterocysts of *Anabaena variabilis*, an experiment that does much to negate the general criticism that such proteins could be derived from eukaryotic contaminants of the cyanobacterial culture.

Using a combination of ion exchange (DEAE-cellulose) and hydrophobic interaction (phenyl-Sepharose) column chromatography, Bianchini *et al.* (1990) achieved a partial purification of putative cyanobacterial calmodulin from *Anabaena* spp. In all, they obtained three polypeptides, one major (58 kDa) and two minor (40 and 16 kDa), all of which were heat stable and capable of activating PDE in the presence of Ca^{2+} . Despite the size of the major protein, the authors suggest that it is the presumptive calmodulin, with the minor proteins being either proteolysis products or isotypes. Notably, the polypeptides were able to stimulate adenylate cyclase obtained from the *Anabaena* spp.

Cyanobacteria are distinguished amongst bacterial species by their use of an O_2 evolving photosynthesis which is similar to and from which, according to the endosymbiotic theory (Margulis, 1981), the chloroplast bound photosynthetic mechanism of higher plants evolved. Both cyanobacterial photosystems (PSI and PSII) require Ca^{2+} (England & Evans, 1983; Becker & Brand, 1985; Piccioni & Mauzerall, 1978; Satoh & Katoh, 1985). The activity of PSII in *A. nidulans* is decreased in parallel with cellular Ca^{2+} in response to increased incident irradiance (England & Evans, *et al.*, 1983; Becker & Brand, 1985). Recently, it has been reported that this Ca^{2+} dependent activity is inhibited by an antibody raised against guinea pig calmodulin (Tranmontini, 1990), which suggests a role for calmodulin in the activation. McColl & Evans (1990) reported the presence of two proteins in *Synechococcus* sp. PCC 7942, (previously known as *Anacystis nidulans*); one of the proteins (~ 60 kDa) was detected with the guinea pig antibody in Western blots of native proteins extracted from thylakoid preparations. The authors suggested that this protein might represent an aggregate of D1 protein, a 32 kDa polypeptide located within the core of the oxygen evolving complex of the PSII particle (Barbock, 1987) and consequently akin to the Ca^{2+} binding protein found in the chloroplast (Koike & Inouye, 1985). When $^{45}\text{Ca}^{2+}$ overlays of the Western blotted proteins were performed, after the method of Maruyama *et al.* (1984), a broad band corresponding to a protein of ~ 60 kDa and a second one of 18 kDa were revealed. The identity of the smaller polypeptide remains unknown, but the authors suggest that it might be analogous to the proteins (24 and 26 kDa respectively) of the chloroplast light harvesting complex, which are known to bind Ca^{2+} (Webber & Gray, 1989).

At Lancaster we have undertaken the purification from *Nostoc* PCC 6720 of a ~ 21 kDa protein that

exhibits many of the characteristics of a calmodulin (Onek, 1991). The protein is essentially acidic as shown by a low isoelectric point ($\text{pI} = 3.9$), poor staining with Coomassie blue and silver stain (though it stains in Western blots with ruthenium red in the procedure of Charuk *et al.*, 1990), and elution at high ion strength from anion exchange chromatography columns. It binds $^{45}\text{Ca}^{2+}$ in the presence of Mg^{2+} in the procedure of Maruyama *et al.* (1984) and shows Ca^{2+} dependent retention on hydrophobic (phenyl-Sepharose) and affinity (fluphenazine-Sepharose) chromatography columns. It exhibits Ca^{2+} dependent activation of both bovine PDE and pea NAD kinase and retains this ability after heating, but not in the presence of calmodulin inhibitors or of the Ca^{2+} chelator EGTA. The purified protein shows poor absorbance at 280 nm and a maximum at 254 nm. It cross reacts with polyclonal antibody preparations raised against spinach calmodulin (Van Eldik & Wolchok, 1984), but not with a commercial preparation raised against bovine brain calmodulin. A polyclonal antibody preparation raised in rabbits against the purified 21 kDa protein cross reacts against spinach calmodulin, but not against bovine brain calmodulin. The latter should not be taken as evidence of a lack of similarity between animal and the putative *Nostoc* calmodulin because the promotion of antibodies to identical epitopes would be expected to be repressed. It demonstrates, however, that both spinach calmodulin and the putative *Nostoc* calmodulin possess epitopes in common that are distinct from those present in the animal calmodulin. To digress momentarily, this finding would, in view of the endosymbiotic theory (Margulis, 1981; Gray & Doolittle, 1982), enhance speculation about the possible presence of a calmodulin in higher plant chloroplasts which may be distinct from the cytosolic form (Jarrett *et al.*, 1982). In addition to the 21 kDa protein, *Nostoc* PCC 6720 contains a 17 kDa protein (Onek *et al.*, 1990) and a 33 kDa protein that bind $^{45}\text{Ca}^{2+}$. The latter also cross reacts with spinach calmodulin and is the predominant protein present in thylakoid membrane extracts.

All four investigations of cyanobacterial calmodulin-like proteins have reported the presence of low molecular mass proteins, with both 17 and 21 kDa proteins shown to be present in *Nostoc* PCC 6720, and the presence of the high molecular mass (~ 58 – 60 kDa) proteins. Two report the presence of mid range (40 and 33 kDa) proteins, the latter being a reasonable candidate for consideration as the D1 protein. Despite native- and SDS-PAGE in the presence and absence of Ca^{2+} there is no evidence from the work in *Nostoc* PCC 6720 to suggest that the 33 kDa is either a dimer of the 17 or 21 kDa proteins or aggregates to form a ~ 60 kDa protein. In addition, the β -subunit of phycocyanin in *Nostoc* PCC 6720 appears to

Table 4. Summary of prokaryotic calmodulin-like proteins

+, Positive reaction; -, negative reaction; n, not reported.

Polypeptide (kDa)	Similarity with calmodulin				Organism	Reference
	Ca ²⁺ binding	Enzyme activation	Immunological cross-reaction	Others		
58-60	+	-	n		<i>E. coli</i>	Harmon <i>et al.</i> (1985)
	+	n	-		<i>Synechococcus</i>	McColl & Evans (1990)
	n	n	n		<i>Anabaena</i>	Bianchini <i>et al.</i> (1990)
40-47	+	-	-		<i>E. coli</i>	Harmon <i>et al.</i> (1985)
	n	+	n		<i>Anabaena</i>	Bianchini <i>et al.</i> (1990)
33	+	n	n		<i>E. coli</i>	Harmon <i>et al.</i> (1985)
	+	n	+		<i>Nostoc</i>	Onek (1991)
20-25	n	+	+		<i>B. subtilis</i>	Fry <i>et al.</i> (1991)
	+	+	+	+*	<i>Nostoc</i>	Onek (1991)
	+	-	-	+†	<i>S. erythraea</i>	Swan <i>et al.</i> (1989)
	+	n	n		<i>B. cereus</i>	Shyu & Foegeding (1991)
16-18	+	n	-		<i>Synechococcus</i>	McColl & Evans (1990)
	+	+	+		<i>Nostoc</i>	Onek <i>et al.</i> (1990)
	n	n	+		<i>Anabaena</i>	Pettersson & Bergman (1989)
	n	+	n		<i>Anabaena</i>	Bianchini <i>et al.</i> (1990)

* Contains Ca²⁺ binding sequence.† Exhibits Ca²⁺ dependent mobility shift on SDS-PAGE.

bind ⁴⁵Ca²⁺ which, together with the natural blue colour of the phycobiliproteins in SDS-PAGE gels (Wood & Haselkorn, 1982), interferes with the detection of the putative *Nostoc* calmodulin by Coomassie blue staining or Ca²⁺ binding.

The summary of prokaryotic 'calmodulin-like' proteins (Table 4) presents a complex picture in which no single protein is readily identified as a calmodulin. In part, this arises from the current lack of complete data on all the proteins reported, but also upon the definition employed which necessarily compares the prokaryotic protein to authentic eukaryotic calmodulin. A physical definition (i.e. amino acid composition/sequence, the presence of conserved epitopes etc.) relies heavily upon genetic conservation. The composition of the putative *B. subtilis* calmodulin does not indicate rigorous conservation and yet it activates Ca²⁺/calmodulin dependent enzymes derived from eukaryotic sources and contains epitopes specifically recognized by an antibody to bovine calmodulin. Plausibly, cyanobacteria may contain more than one form of calmodulin as well as PSII Ca²⁺ binding proteins. The existence of calmodulin isotypes is known: the eggs of the sea urchin (*Arbacia punctulata*) contain two isotypes (Burgess, 1982). Named calmodulin A and calmodulin B, the two isotypes differ in their electrophoretic mobility, molecular mass (A, 20-21 kDa; B, 16.5-17.5 kDa), amino acid composition, carboxy-terminal amino acid sequence, and reactivity with site specific anti-vertebrate antibodies (Burgess, 1982). Further work is required to resolve the various questions.

Even so, the weight of evidence at present supports the view that bacteria contain proteins capable of acting as signal transducing components of Ca²⁺ mediated regulatory mechanisms.

Calcium mediated regulation in prokaryotes

The demonstration of prokaryotic calmodulin-like proteins begs the question of which metabolic and physiological processes in the prokaryotic cell might be regulated through calcium/calmodulin dependent mechanisms. The question is not easily put either by reference to the literature or experimentally. There is some preliminary work on cyanobacterial processes, but very little published work on other bacteria. Fry *et al.* (1991), in their work on the *B. subtilis* calmodulin-like protein report that sporulation is blocked by the calmodulin antagonists promethazine and trifluoperazine. Calcium is known to be required for sporulation in several bacterial species (Campbell, 1983) and in *B. subtilis* the requirement includes the efficient protein degradation which accompanies the process (Green & Slepecky, 1972). Encystment in bacterial species such as *Azotobacter* also requires Ca²⁺ and mature cysts rupture if incubated in calcium depleted media (Page & Sadoff, 1975).

Heterocyst cytodifferentiation is also influenced by Ca²⁺. The heterocyst is a specialized cell that develops from the vegetative cells of some filamentous species of cyanobacteria when they are deprived of combined

nitrogen. It protects the labile N_2 fixing complex, nitrogenase, from O_2 (Haselkorn, 1978). In parallel with *B. subtilis* sporulation, protein degradation accompanies heterocyst development. This was thought to involve a calcium requiring serine protease that has been identified in *Anabaena* sp. However, replacement of the wild-type gene by a dysfunctional form has recently shown that the protease is dispensable (Maldener *et al.*, 1991). In *Nostoc* PCC 6720 the proportion of heterocysts to vegetative cells in the culture is correlated with treatments which affect the cellular calcium content as estimated by $^{45}Ca^{2+}$ labelling experiments (Smith *et al.*, 1987a, b). At low light intensities the effect has been related to the incident irradiance upon these phototrophic organisms such that as light is decreased the proportion of heterocysts and the cellular Ca^{2+} content increase (Smith & Wilkins, 1988). Calmodulin antagonists appear to have little effect on either the differentiation of heterocysts or the cellular content of calcium in *Nostoc* 6720 (Onek *et al.*, 1991). However, Zhao *et al.* (1991) report that the antagonist W7, or calcium depletion, double the proportion of heterocysts in *Anabaena* 7120 cultures, while the calcium ionophore calcimycin (compound A23187), in the presence of calcium, completely inhibits heterocyst differentiation if applied prior to the induction of heterocyst formation by the removal of nitrate from the medium.

Diazotrophy (Wilson, 1971; Norris & Jensen, 1957) and N_2 fixation in cyanobacteria (Eyster, 1972) require Ca^{2+} . Calcium is known to be important in a mechanism which affords protection to the nitrogenase complex from O_2 in both unicellular (Hamadi & Gallon, 1981; Gallon & Hamadi, 1984) and filamentous species (Rodriguez *et al.*, 1990). Calmodulin inhibitors also inhibit nitrogenase activity in the presence of O_2 and decrease the ability of an O_2 stressed culture to recover N_2 fixation (Onek, 1991). However, calcium ionophores, which increase cellular calcium, depress acetylene reduction (Smith *et al.*, 1987a, b). Lanthanum (Smith *et al.*, 1987a, b) and calmodulin inhibitors (Onek *et al.*, 1991), when added to the growth media at low concentrations, are able to stimulate acetylene reduction, doubling the rate under certain conditions. These findings suggest that nitrogenase is susceptible to a second calcium mediated process which is distinct from the O_2 protection mechanism and normally depresses N_2 fixation below the maximal rate attainable by the culture. There is no evidence to suggest Ca^{2+} has any direct influence on the nitrogenase complex, but a mechanism which influences metabolic processes peripheral to N_2 fixation, such as the supply of reductant or ATP, is feasible.

England & Evans (1983) have shown that the activity of PSII preparations from *Synechococcus* PCC 7942 is

inhibited if Ca^{2+} is removed and may be restored by restoration of Ca^{2+} and Na^+ . The guinea pig calmodulin antibody, which detects the 60 kDa protein in Western blots, prevents this reactivation. Although the 60 kDa protein is difficult to reconcile with the D1 intrinsic core protein, the involvement of the latter was supported by the recent finding that a *Synechococcus* PCC 7942 mutant that has a chromosomal deletion including the *psbA1* gene (the major D1 protein gene) grew poorly on low Ca^{2+} medium and yielded PSII preparations that cannot be reactivated after Ca^{2+} depletion. In *in vitro* protein phosphorylation studies of PSII preparations, proteins of 12 and 14 kDa are heavily labelled, but they are absent in the mutant and replaced by an anomalous phosphorylation of a 10 kDa protein (H. Evans, personal communication).

Concluding remarks

Experimentally, a demonstration of the existence of calcium mediated regulation in bacteria requires a step-by-step analysis of signal transduction from stimulus to response. It is relatively simple to demonstrate a calcium requirement in a metabolic process (indeed the literature abounds with calcium requiring bacterial processes which may, in some instances, reflect calcium mediated regulation; viz. Smith, 1988). Demonstrating that a correlation exists between a technique which varies cellular Ca^{2+} and a metabolic or physiological process is hardly more difficult. While such experiments are useful indicators of potential for Ca^{2+} regulated processes, they provide only circumstantial evidence for calcium mediated regulation. The identification of putative bacterial calmodulins provides evidence for calcium mediated regulation on the basic assumption that they would not be present were they not required. More important to the cause is that the putative calmodulins demonstrate a possible means of Ca^{2+} signal transduction in the bacteria.

In identifying stimuli of Ca^{2+} mediated regulation in bacteria the lack of information makes it difficult to decide where to look. As a working hypothesis we favour the idea that Ca^{2+} mediated regulation may be monitoring the carbon/nitrogen balance or energy state of the cell. This hypothesis would concur with the one stimulus in phototrophic cyanobacteria, incident irradiation, that we have identified (Smith & Wilkins, 1988). This hypothesis is also tolerable when those cyanobacterial processes (i.e. PSI and PSII, N_2 fixation and heterocyst differentiation, phosphate uptake and the phototactic response) and bacterial processes (i.e. sporulation) that

preliminary evidence suggests to be under some Ca^{2+} mediated regulation are considered. The cell could be expected to benefit if these processes were influenced by such a mechanism. The known regulatory roles of cyclic nucleotides and the presence of a Ca^{2+} /calmodulin dependent adenylate cyclase (Bianchini *et al.*, 1990) offer further support.

A fundamental requirement for Ca^{2+} -mediated regulation is the ability of the species in question to regulate intracellular calcium. Considering the cytotoxic effects of excess Ca^{2+} , all cells are likely to possess the means of expelling Ca^{2+} and therefore maintaining a Ca^{2+} concentration gradient across the cell membrane. In bacteria, antiporters coupling Ca^{2+} efflux to Na^+ and H^+ gradients have been reported (Rosen, 1986) as well as Ca^{2+} dependent Mg^{2+} -ATPases (*Streptococcus faecalis*, Kobayashi *et al.*, 1978; *Anabaena* sp., Lockau & Pfeffer, 1983). Ca^{2+} influx may involve Ca^{2+} channels. Studies on the phototactic response of cyanobacteria have included Ca^{2+} channel blocking agents such as ruthenium red (Haeder, 1982), which imply their existence. Using the crude method of $^{45}\text{Ca}^{2+}$ long-labelling, the effects of various treatments on heterocyst proportion and N_2 fixation in *Nostoc* PCC 6720 have been related to changes in the intracellular Ca^{2+} content (Smith *et al.*, 1987a, b; Smith & Wilkins, 1988). Such crude methods do not describe the important factor of cytosolic free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, but the single report of using a fluorescent dye to estimate $[\text{Ca}^{2+}]_i$ in a bacterium (Gangola & Rosen, 1987) emphasizes the difficulties of the technique. An elegant solution has been constructed in the form of a recombinant *E. coli* clone expressing the protein aequorin, which emits blue light on binding Ca^{2+} . Reconstitution *in vivo* with the prosthetic group of the protein, coelenterazine, provides a direct, non-perturbing estimation of $[\text{Ca}^{2+}]_i$ (Knight *et al.*, 1991a). In this way *E. coli* has been shown to respond to changes in the external Ca^{2+} concentration with a brief increase in $[\text{Ca}^{2+}]_i$ followed by a rapid (<30 s) re-establishment of the steady state $[\text{Ca}^{2+}]_i$ (Knight *et al.*, 1991a). Thus, the potential for transducing Ca^{2+} signals through a change in the membrane calcium gradient is present. The technique promises rapid progress not only in bacteria, but also in higher plants (Knight *et al.*, 1991b).

In establishing stimulus response coupling there remains the need to demonstrate that the bacterial calmodulins respond to changes in $[\text{Ca}^{2+}]_i$ within the physiological range by activating or repressing enzymic activities. The only bacterial enzyme so far reported to be Ca^{2+} /calmodulin dependent is adenylate cyclase. This dependence has been shown for the enzyme obtained from such diverse prokaryotic sources as *Anabaena* sp. (Bianchini *et al.*, 1990), *Bordetella pertussis* (Wolff *et al.*, 1980; Gordon *et al.*, 1989) and *Bacillus anthracis* (Leppla,

1982; Gordon *et al.*, 1989). In the latter two cases, the activity of adenylate cyclase is directly connected with the virulence of these organisms in causing whooping cough and anthrax, respectively, and thus the involvement of the Ca^{2+} /calmodulin system has important implications for the complete understanding of their pathogenesis. Further work, perhaps guided by the known Ca^{2+} /calmodulin dependent enzymes in eukaryotes, is required to extend the bacterial repertoire. The finding of calmodulin-like proteins and Ca^{2+} binding domains in bacteria, the presence of Ca^{2+} pumps and the maintenance of $[\text{Ca}^{2+}]_i$, and the accumulating evidence of regulated bacterial processes all substantiate the concept of Ca^{2+} mediated regulation in the bacterial cell. In doing so they also indicate that the mechanism first evolved in bacteria, and later was refined and adapted to the various purposes of eukaryotes. Consequently, it is not unlikely that bacteria will contain a simpler version of many of the regulatory mechanisms found in eukaryotes. Calcium mediated regulation of protein phosphorylation would be a good example. The first indications that this exists have been reported by Mann *et al.* (1991) and H. Evans (personal communication). Equally suggestive is the presence in cyanobacteria (Hirsch *et al.*, 1989) of the plant regulator abscisic acid (ABA), which has recently been shown to increase $[\text{Ca}^{2+}]_i$ in the guard cells of stomata and so contribute to the regulation of water balance in higher plants (Hetherington & Quatrano, 1991). ABA affects growth, heterocyst differentiation and N_2 fixation in cyanobacteria (Marsalek *et al.*, 1991; Smith *et al.*, 1987b) in a manner comparable to that of a calcium ionophore, and results in a detectable increase in the intracellular calcium content of *Nostoc* PCC 6720 (Smith *et al.*, 1987b).

In conclusion, it is clear that while the available evidence for the concept is persuasive, the extent of Ca^{2+} mediated regulation in bacteria has yet to be assessed. Irrespective of whether it proves to be a minor or a major factor, the investigation has begun – which for many of us is the most enjoyable part.

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