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Comparison of the NAD Kinase and Myosin Light Chain Kinase Activator Properties of Vertebrate, Higher Plant, and Algal Calmodulins[1]

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ABSTRACT

In the preceding paper (Lukas, Iverson, Schleicher, Watterson 1984 Plant Physiol 75: 788–795), we reported that the amino acid sequence of spinach calmodulin has at least 13 amino acid sequence differences from vertebrate calmodulin. In the present study, we investigated the effect of these amino acid sequence substitutions on the enzyme activator properties of vertebrate and plant calmodulins. Calmodulins from spinach and the green alga *Chlamydomonas reinhardtii* activate chicken gizzard myosin light chain kinase in a manner similar but not identical to chicken calmodulin. In contrast, these calmodulins have very different NAD kinase activator properties. The concentration required for half-maximal activation of pea seedling NAD kinase by spinach calmodulin (3–4 nanomolar) is lower than the corresponding concentrations of chicken (20 nanomolar) and *Chlamydomonas* (40 nanomolar) calmodulins. However, the maximum level of activation obtained with *Chlamydomonas* calmodulin is 4- to 6-fold higher than spinach or chicken calmodulin. These data indicate that the limited structural heterogeneity among calmodulins have differential effects on their biochemical activities.

In the preceding paper (12), we demonstrated that the amino acid sequence of spinach calmodulin is highly conserved and differs from the bovine brain calmodulin sequence (6, 22) in only 13 of 148 residues. The high degree of structural homology between plant and vertebrate calmodulin is paralleled by similarities in certain biochemical properties. For example, the phosphodiesterase activator properties of a variety of plant calmodulins, including those from spinach (21), barley (16), zucchini (13), peanut (2), and the green alga *Chlamydomonas* (17), are similar, if not identical, to the activator properties of vertebrate calmodulin. In contrast, a substantial difference in the NAD kinase activator properties of peanut and porcine brain calmodulins has been reported (8). Thus, it appears as if the limited amino acid sequence differences between higher plant and vertebrate calmodulins may have important functional significance.

In the present study we have extended our investigation of calmodulin structure and function by comparing the properties of higher plant, vertebrate, and algal calmodulins as activators of vertebrate MLCK[2] and plant NAD kinase.

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[2] Abbreviations: MLCK, myosin light chain kinase; EGTA, ethylene bis(oxyethylenenitrilo)tetraacetic acid; MOPS, 2-(*N*-morpholino)propanesulfonic acid.

MATERIALS AND METHODS

Materials. Pea seeds (*Pisum sativum* var Early Alaska) were obtained from Ernest Hardison Seed Co., Nashville, TN. NAD and NADP were obtained from Sigma Co. ATP was obtained from Boehringer-Mannheim, West Germany. γ-^{32}P-ATP was prepared as previously described (19). All other chemicals were reagent grade.

Preparation of Proteins. Calmodulin was isolated from chicken gizzard (5), spinach leaves (12), and *Chlamydomonas reinhardtii* (17) essentially as previously described. In the case of higher plant and algal tissues, calmodulin was extracted in the presence of polyvinylpolypyrrolidone as described previously (16, 17) to prevent protein modification by phenolic compounds. The purity of all calmodulin preparations was assessed by SDS-gel electrophoresis and by amino acid composition analyses. MLCK was purified from chicken gizzards by using the procedure described by Adelstein and Klee (1). Gel electrophoretic analysis of the purified MLCK preparation resolves one major protein band (apparent M_r = 130,000) and two minor bands (apparent M_r = 115,000 and 105,000). All bands bind to [^{125}I]calmodulin and show immunoreactivity with anti-MLCK antibodies (data not shown).

Mixed light chains were prepared from bovine cardiac muscle myosin by the method described by Blumenthal and Stull (4). The regulatory light chain was purified by applying mixed light chains (in 10 mM MOPS, 15 mM 2-mercaptoethanol, pH 7.0) to an affigel blue (Bio-Rad) column that was equilibrated in the same buffer. The column was washed, and regulatory light chains were eluted with a linear gradient of 0 to 0.4 M KCl in 10 mM MOPS, 15 mM 2-mercaptoethanol, pH 7.0.

NAD Kinase Extraction and Assay. NAD kinase was extracted and partially purified using modifications of previously described procedures (8, 15). Two-week-old pea seedlings (100 g) were homogenized in 300 ml of 25 mM triethanolamine HCl (pH 7.5), 0.5% (w/v) polyvinylpolypyrrolidone, and 1 mM phenylmethylsulfonylfluoride. The homogenate was squeezed through cheesecloth and centrifuged at 12,000*g* for 30 min. NAD kinase in the supernatant was purified through the protamine sulfate and PEG precipitation steps as described by Muto and Miyachi (15). The precipitate obtained from the PEG step was dissolved in 50 mM Tris HCl (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA and was passed through a 4- × 1-cm DEAE Sephadex A-25 column. The effluent contained NAD kinase activity that was completely dependent upon Ca and calmodulin for activity. the partially purified NAD kinase was stored frozen at −80°C until use.

NAD kinase activity was assayed by a modification of the method of Wang and Kaplan (20) as described by Muto and

Miyachi (15). The assay mixture contained 3 mM ATP, 2 mM NAD, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM KCl, 50 mM Tris HCl (pH 8.0), and various amounts of calmodulin. The assay was initiated by the addition of NAD kinase, and incubation was carried out at 37°C for 30 min. The assay was terminated by boiling for 5 min and the NADP produced ws measured by the method of Apps (3).

Assay of MLCK Activator Activity. Enzyme activity was determined by the incorporation of ^{32}P into the regulatory light chain of cardiac muscle myosin. Reaction mixtures (50 μl final volume) contained 45 μM light chain, 25 mM MOPS (pH 7.0), 10 mM magnesium acetate, 0.2 mM $CaCl_2$, 7.5 mM 2-mercaptoethanol, 1 mM [γ-^{32}P]ATP (150 cpm/pmol), and 1.5 nM chicken gizzard MLCK. The assays contained varying amounts of calmodulin from chicken gizzard, spinach or *Chlamydomonas* as indicated in the legend to Figure 1. Some assays also contained 1 mM EGTA as indicated. Reactions were initiated by the addition of [γ-^{32}P]ATP and transfer to a 30°C water bath. Aliquots (25 μl) of the reaction mixtures were removed after 20 min and spotted onto squares of phosphocellulose paper. The squares were allowed to dry for 2 min and were then washed in 75 mM H_3PO_4 (five washes, 5 min each, 10 ml/square). The squares were washed once in 95% ethanol, dried, and suspended in Betafluor (National Diagnostics, NJ) for determination of radioactivity.

Protein Determination. Protein concentration was determined by the method of Lowry (11) using BSA as a standard. Calmodulin concentration was determined by amino acid composition (18).

RESULTS AND DISCUSSION

The ability of various calmodulin preparations to activate MLCK was examined (Fig. 1). The specific activity of MLCK in the presence of Ca with no added calmodulin is 20 nmol of phosphate incorporated into myosin light chains/min·mg protein. In the presence of EGTA, the specific activity of the kinase is 13 nmol/min·mg protein. At saturating concentrations of vertebrate calmodulin, the activity reaches 980 nmol/min·mg protein. This value is in agreement with that obtained by Adelstein and Klee for turkey gizzard MLCK (1). Neither spinach calmodulin nor *Chlamydomonas* calmodulin is able to activate

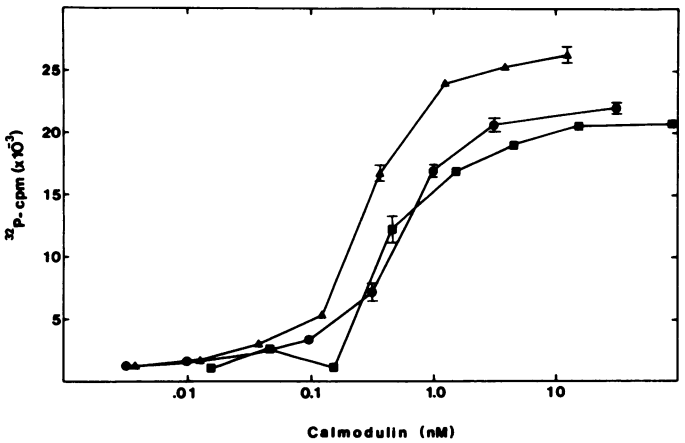

FIG. 1. MLCK activator activities of chicken gizzard, spinach, and *Chlamydomonas* calmodulins. Assays were performed as described in "Materials and Methods." The effects of increasing concentrations of calmodulin from chicken gizzard (▲), spinach (●), and *Chlamydomonas* (■) on the incorporation of ^{32}P into the regulatory light chain of cardiac myosin are shown. Each point is the average of duplicate determinations. The error bars show the range of values obtained. The absence of error bars indicates that the range is smaller than the size of the symbol.

MLCK to the same specific activity obtained with vertebrate calmodulin. The maximal activity of MLCK obtained with saturating concentrations of spinach and *Chlamydomonas* calmodulin is approximately 20% less than that obtained with vertebrate calmodulin. There also are minor but reproducible differences in the amount of the various calmodulins required for half-maximal activation of MLCK. Half-maximal activation of MLCK is obtained between 0.2 and 0.4 nM for all calmodulins tested.

The same calmodulin preparations used in the MLCK activator assays were tested for NAD kinase activator activity. In contrast to the data obtained with myosin light chain kinase, the differences in the NAD kinase activator properties of spinach, *Chlamydomonas*, and vertebrate calmodulin are more striking. The concentration of spinach calmodulin required for half-maximal activation of NAD kinase is 3 to 4 nM (Fig. 2A). This concentration is significantly lower than the corresponding values obtained for vertebrate (20 nM) and *Chlamydomonas* (40 nM) calmodulin (Fig. 2, A and B). Algal, higher plant, and vertebrate calmodulins are also readily distinguishable by the degree of NAD kinase activation observed with saturating concentrations of calmodulin. The highest activation of NAD kinase was obtained with *Chlamydomonas* calmodulin (Fig. 2B) while the maximum activations obtained with spinach and vertebrate calmodulins were 4-fold and 6-fold lower, respectively (Fig. 2, A and B). The activation of NAD kinase by the three calmodulin preparations is completely inhibited by the substitution of 1 mM EGTA for calcium in the assay.

The 5- to 6-fold difference between the concentrations of chicken and spinach calmodulin required for half-maximal activation is consistent with that observed by Jarrett *et al.* (8) with peanut and porcine calmodulins. However, in contrast to the results in our study, Jarret *et al.* (8) found no apparent differences in the maximal activation of NAD kinase by higher plant and vertebrate calmodulins. Algal calmodulin was not tested. Although the reason for this discrepancy is not known, it may be related to differences in the enzyme preparation used. The purification and characterization of NAD kinase are necessary before an understanding of these differences can be obtained.

Amino acid sequence analyses of spinach (12) and *Chlamydomonas* (17) calmodulins indicate that they share a high degree of structural homology with vertebrate calmodulin, but also have a limited number of unique amino acid substitutions. Despite these structural differences, spinach and *Chlamydomonas* calmodulins activate cyclic nucleotide phosphodiesterase (17, 21) and MLCK in a manner similar to that of vertebrate calmodulin. These results indicate that highly conserved regions of the calmodulin molecule are involved in the activation, or that structural changes in the protein can occur without significant alteration of MLCK or cyclic nucleotide phosphodiesterase activator activities. However, the differences in the activation of NAD kinase by the same calmodulin preparations indicate that these limited structural differences can influence the functional properties of the protein. It remains to be determined whether NAD kinase utilizes a functional domain on the calmodulin molecule that is distinct from those utilized in phosphodiesterase or MLCK activation, or whether NAD kinase interacts at the same sites as MLCK and phosphodiesterase but has a stricter structural requirement for activation. A previous example of how limited amino acid sequence changes in calmodulin can result in modified protein binding properties has been reported by Van Eldik and co-workers (7), who demonstrated that single amino acid substitutions can result in substantial decreases in immunoreactivity of calmodulin with certain anti-calmodulin antibodies.

It could be argued that the differences observed in the NAD kinase activator properties of the three calmodulin preparations may be the result of modification by secondary metabolites or

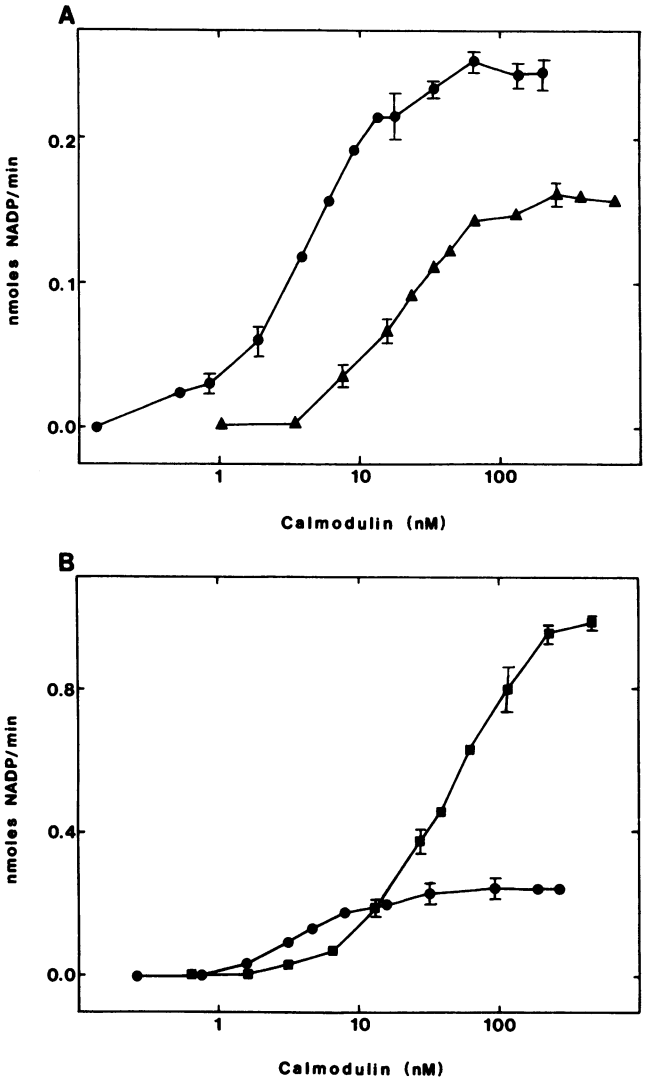

FIG. 2. NAD kinase activator activities of chicken gizzard, spinach, and *Chlamydomonas* calmodulins. NAD kinase activity was assayed as described in "Materials and Methods." A, Activator curves for spinach (●) and chicken gizzard calmodulins (▲). B, Activator curves for spinach (●) and *Chlamydomonas* (■) calmodulins. Each point is the average of duplicate determinations. The error bars show the range of values obtained. The absence of error bars indicates that the range is smaller than the size of the symbol.

proteases during their extraction and isolation. As previously described (12, 16, 17), the protocols used for the extraction and isolation of plant and algal calmodulin were designed to prevent modification by secondary metabolites. In addition, the calmodulins used in these studies have been extensively characterized, including by amino acid sequence analyses, and evidence of modification was not obtained (12, 17, 18, 21). Finally, we have recently discovered that calmodulin from the microorganism *Dictyostelium discoideum* activates NAD kinase in a manner similar to that of *Chlamydomonas* (14). Thus, if the activation differences are the result of the modification of calmodulin during extraction and isolation, these modifications are not readily detected and are common to calmodulins from plant and non-plant species.

Differences in the activator properties of calmodulins from different organisms have been observed with calmodulin-sensitive enzymes other than NAD kinase. Kakiuchi *et al.* (9) reported a guanylate cyclase activity in *Tetrahymena* which was stimulated by *Tetrahymena* calmodulin but not by vertebrate or invertebrate calmodulins. Recently, Klumpp *et al.* (10) demonstrated a calmodulin-sensitive guanylate cyclase activity in *Paramecium*. They found that the enzyme was stimulated by vertebrate, higher plant, *Tetrahymena*, and *Paramecium* calmodulins, but not by *Dictyostelium* calmodulin.

The results summarized here and in the accompanying report (12) demonstrate that the limited number of conservative amino acid differences between vertebrate and plant calmodulins can have functional significance. The work presented here, combined with previous reports from other laboratories (8–10), suggest that certain enzymes are selective in their interactions with different calmodulins, and emphasize the importance of detailed structural analyses of different calmodulins and the proteins with which they interact.

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