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BINDING OF PROTEIN KINASE SUBSTRATES BY FLUORESCENTLY LABELED CALMODULIN

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SUMMARY: A synthetic protein kinase substrate, PRO-LEU-SER-ARG-THR-LEU-SER-VAL-SER-SER-NH_2, undergoes calcium-dependent binding by calmodulin. Phosphorylation of the peptide decreases its affinity for calmodulin with the dissociation constant increasing from 2.4 to ca. 7 mM. The results are consistent with the suggestion that calmodulin and the cAMP-dependent protein kinase can act on common recognition sequences.

INTRODUCTION

Calmodulin is the major intracellular receptor of calcium. It is involved in the regulation of diverse cellular functions. The binding of calcium stabilizes a specific conformation of the calmodulin molecule recognized by calmodulin-dependent enzymes such as the cyclic nucleotide phosphodiesterase, adenylate cyclase, and myosin light chain kinase (reviewed by Cheung in Ref. 1). Calmodulin also shows calcium-dependent binding of small molecules including phenothiazine drugs (2), the fluorescent dye 9-anthroylcholine (3), and several hormones and neuropeptides (4,5). Regardless of the physiological relevance of the latter interactions, they may provide clues concerning the binding specificity of calmodulin. Malencik and Anderson (5) found that the peptide hormones and neurotransmitters which calmodulin binds well (ACTH, β-endorphin, substance P, and glucagon) contain sequences which are similar to the recognition sequence for the cAMP-dependent protein kinase except for the absence of phosphorylatable

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serine and threonine residues. Sequenced phosphorylation sites in physiologically significant cAMP-dependent protein kinase substrates often follow the patterns ARG-ARG-X-SER and LYS-ARG-X-X-SER (6,7) with hydrophobic residues flanking the C-terminal side of the phosphorylated serine (8).

This paper describes experiments with simple protein kinase substrates to test the hypothesis that calmodulin and protein kinase can act on common recognition sequences with effects of peptide phosphorylation on calmodulin binding. The peptides examined are Kemptide (LEU-ARG-ARG-ALA-SER-LEU-GLY), the widely used synthetic protein kinase substrate (9), and the synthetic decapeptide corresponding to the N-terminal sequence of glycogen synthase (PRO-LEU-SER-ARG-THR-LEU-SER-VAL-SER-SER-NH_2). The latter is phosphorylatable at position 7 by either the cAMP-dependent protein kinase or phosphorylase kinase (10).

MATERIALS AND METHODS

Porcine calmodulin was prepared by the procedure of Schreiber et al. (11) and the catalytic subunit of beef heart cAMP-dependent protein kinase, according to Peters et al. (12). Kemptide was purchased from Sigma Chemical Co. The synthetic glycogen synthase decapeptide was a gift from Prof. Bruce Kemp and the α-melanocyte stimulating hormone a gift from Prof. A. B. Lerner. Dansyl calmodulin was previously prepared and characterized by us (5,13). It binds small peptides (5) and smooth muscle myosin light chain kinase (13) as well as does unmodified calmodulin. The dissociation constants obtained with labeled and native calmodulin agree within experimental error. Fluorescence measurements were carried out with the Hitachi-Perkin Elmer MPF-2A fluorometer. Data were analyzed as previously described (5,14). Our buffer contained 50 mM MOPS, 0.2 M KCl, and 1.2 mM $Ca(CH_3CO_2)_2$ dissolved in glass distilled H_2O (pH 7.3).

RESULTS

Malencik and Anderson showed that the fluorescence of dansyl calmodulin is exceptionally responsive to both calcium and protein binding. The binding of specific peptides and proteins such as smooth muscle myosin light chain kinase or troponin I by dansyl calmodulin causes the fluorescence quantum yield to increase from 0.25 to 0.35-0.45 and the emission maximum to shift from 525 to 505 nm (5). Figure 1 shows the increase in relative fluorescence intensity (F/F_o) occurring on the titration of a 10 μM solution of dansyl calmodulin with PRO-LEU-SER-ARG-THR-LEU-SER-VAL-SER-SER-NH_2. The corresponding dissociation constant obtained from a double reciprocal plot

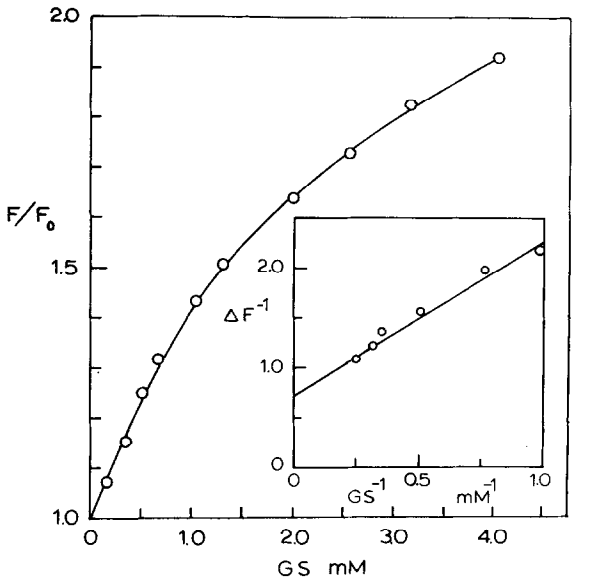

Fig. 1. Fluorescence titration of 10 μM dansyl calmodulin with the glycogen synthase decapeptide, PRO-LEU-SER-ARG-THR-LEU-SER-VAL-SER-SER-NH_2. The fluorescence enhancement (F/F_o) was measured at 460 nm, with 340 nm excitation. Conditions: 50 mM MOPS, 0.2 N KCl, 1.2 mM $Ca(CH_3CO_2)_2$, pH 7.3, 25°.

of the changes in fluorescence versus the peptide concentration is 2.4 mM and the fluorescence enhancement factor at saturation (F_∞/F_o) is 2.5. Similar titrations in solutions containing 1.2 mM EDTA and no added Ca^{++} showed no fluorescence change.

Next we performed an experiment to see whether phosphorylation of the decapeptide has an effect on its binding to calmodulin. We added 5.0 mM ATP, 6.0 mM $MgCl_2$, and 1.0 mM dithiothreitol to a solution containing 9.2 μM dansyl calmodulin and 4.0 mM of the decapeptide substrate. The fluorescence was unaffected by these additions. At zero time, we added 4 μl per ml of a 5 mg/ml solution of the catalytic subunit of protein kinase. The fluorescence of the mixture declined linearly for the first 5-10 min and leveled off after 30 min (Fig. 2). Addition of 1.4 mM ATP to the equilibrium mixture caused no further decrease, showing completeness of the phosphorylation reaction. Since the peptide concentration (X) used was non-saturating, the fluorescence should respond to either increased or decreased binding of the peptide. Increased peptide binding would cause an increase in F/F_o, with a maximum possible value of 2.5, while total

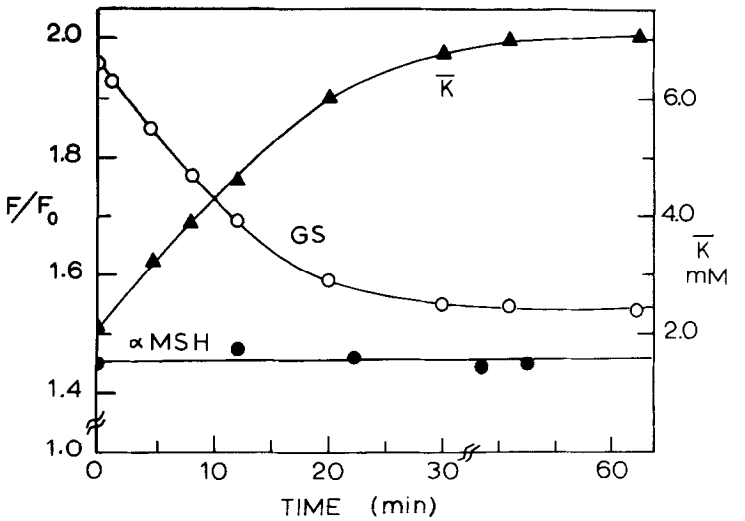

Fig. 2. Effect of phosphorylation on the binding of the glycogen synthase decapeptide (4.0 mM) by dansyl calmodulin (9.3 μM). At zero time, the catalytic subunit of protein kinase was added to give ∿20 μg/ml. The decline in fluorescence enhancement (O), the average dissociation constants calculated at various times (▲), and a control experiment using α-melanocyte stimulating hormone (100 μM) instead of the decapeptide (●) are shown. F/F_o was measured at 460 nm for the decapeptide and at 450 nm for α-MSH. Conditions: as in Fig. 1, plus 6.0 mM $MgCl_2$, 5.0 mM ATP, and 1.0 mM dithiothreitol.

dissociation of the complex would result in $F/F_o = 1$. The fluorescence enhancement at equilibrium ($F/F_o = 1.54$) shows that partial dissociation had occurred. Since the fluorescence enhancement is related to the fractional saturation (ϕ) of calmodulin with the peptide [$\phi = (F/F_o-1)/(F_\infty/F_o-1)$], the change in ϕ and in the dissociation constant [$K = (1-\phi)X/\phi$] can be calculated (14). The time course in Fig. 2 is consistent with a decrease in ϕ, from 0.63 to 0.36, and with an increase in K from 2.3 to 7.1 mM.

We performed control experiments to rule out phosphorylation of calmodulin as the cause of the fluorescence change. We incubated calmodulin alone in the phosphorylation mixture. No change in initial peptide binding occurred after 45 min of incubation. In a second experiment, we attempted phosphorylation in the presence of the α-melanocyte stimulating hormone, which contains no phosphorylatable residues and also binds calmodulin (K_d = 59 μM) with fluorescence enhancement (1.7 fold maximum) (5). The ratio of the α-MSH concentration to the dissociation constant was fixed at 1.7, matching the ratio used with the decapeptide. Incubation in the

phosphorylation mixture had no effect on the binding of α-MSH by calmodulin, showing that the binding properties of calmodulin are unchanged.

Kemptide (LEU-ARG-ARG-ALA-SER-LEU-GLY) also binds calmodulin, although at least an order of magnitude less well than the decapeptide, and shows effects of peptide phosphorylation on calmodulin binding.

DISCUSSION

These experiments support the hypothesis that calmodulin and the cAMP-dependent protein kinase can act on a common polypeptide sequence with effects of peptide phosphorylation on calmodulin binding (5). The fluorescence decrease accompanying phosphorylation of the model decapeptide is consistent with increased dissociation of the peptide-dansyl calmodulin complex. The apparent dissociation constant increases from 2.3 to 7.1 mM. Quantum yield changes may also occur; however, the extrapolated fluorescence enhancement factor obtained with the phosphorylated peptide is about the same as that obtained with the unphosphorylated peptide (data not shown). Known changes in calmodulin binding following phosphorylation occur in smooth muscle myosin light chain kinase, with a 20 (15) to 500 (13) fold decrease in affinity, and in nonenzymic calmodulin binding proteins including troponin I, histone H2A, and the myelin basic protein with 2 to 5 fold decreases in affinity (13). The moderate effects of phosphorylation on the binding of calmodulin by either the model peptide or the latter three proteins probably result from the changes in local side chain interactions. The much larger effect obtained on phosphorylation of myosin light chain kinase may reflect more long range conformational changes. Phosphorylation often occurs in the N- or C-terminal regions of the affected proteins. Phosphorylation of phosphorylase results in a more rigid association of the N-terminal region with the compact mass of the enzyme molecule (16), possibly demonstrating a type of conformational change common in phosphorylated proteins.

Although the decapeptide is derived from an enzyme which is not calmodulin-dependent, glycogen synthase, the arginine and valine residues

occupy the same positions relative to the phosphorylated serine residue often found in calmodulin binding proteins (5). The fact that it binds calmodulin 10^3 fold less well than the hormones and neuropeptides previously studied reflects the absence of the additional basic and hydrophobic residues in the ideal model envisioned, which contains a strongly basic tripeptide sequence three positions away from a pair of bulky hydrophobic residues (5). The affinities of calmodulin, the cAMP-dependent protein kinase, and phosphorylase kinase for the decapeptide must be comparable since our K_d is close to the K_m's reported for the two kinases and the corresponding pentadecapeptide (10). The weak binding of Kemptide by calmodulin is attributable to its small size and the absence of suitable hydrophobic residues. Our results with the peptides suggest that the parent enzymes - glycogen synthase and pyruvate kinase - may also interact with calmodulin to some extent.

We are now working on the preparation of low molecular weight calmodulin binding peptides from calmodulin binding proteins with phosphorylatable serine residues (smooth muscle myosin light chain kinase, troponin I, and the myelin basic protein) and from calcineurin. Experiments with these peptides will demonstrate whether calmodulin recognizes phosphorylatable sequences from calmodulin binding proteins and whether peptide phosphorylation affects the interaction. The techniques developed here and in our initial studies with peptide hormones and neurotransmitters (5) will be applied.

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