Association of Calmodulin with Peptide Analogues of the Inhibitory Region of the Heat-Stable Protein Inhibitor of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

Dean A. Malencik,‡ John D. Scott,§ Edmond H. Fischer,‖ Edwin G. Krebs,§ and Sonia R. Anderson*,‡

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, and Department of Pharmacology and Howard Hughes Medical Institute and Department of Biochemistry, University of Washington, Seattle, Washington 98195

Received December 31, 1985; Revised Manuscript Received February 21, 1986

ABSTRACT: A 20-residue peptide analogue (IASG*R*TG*RR*NAIHDILVSSA) of the 8000-dalton heat-stable cAMP-dependent protein kinase inhibitor undergoes efficient calcium-dependent binding by calmodulin, with $K_d \sim 70$ nM when calcium is present. It is a potent inhibitor of smooth muscle myosin light chain kinase and of the calmodulin-dependent phosphatase activity of calcineurin. At concentrations above 3 μM, the peptide stimulates the basal activity of calcineurin. The native protein kinase inhibitor has no effect on the catalytic activity of myosin light chain kinase and is moderately inhibitory to both the calmodulin-dependent and -independent phosphatase activity of calcineurin. Competition experiments using excess concentrations of calcineurin and calmodulin suggest that the primary interaction of the native heat-stable inhibitor is with the catalytic subunit of protein kinase. Dansylcalmodulin exhibits only a weak interaction with the inhibitor. Observations on deletion peptides of the 20-residue analogue help to delineate the overlapping peptide binding specificities of the cAMP-dependent protein kinase [Scott, J. D., Glaccum, M. B., Fischer, E. H., & Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1613–1616] and calmodulin. In both cases, the most effectively bound peptides contain the *R*TG*RR* sequence.

Calmodulin and the cAMP-dependent protein kinase are the major intracellular receptors for the second messenger molecules, calcium (Ca^{2+}), and cAMP. That there are several levels of interaction between these signalling systems is well-known. For example, the enzymes catalyzing cAMP synthesis and degradation, adenylate cyclase and cyclic nucleotide phosphodiesterase, are both activated by the calcium–calmodulin complex (Cheung, 1971, 1981; Cheung et al., 1975; Brostrom et al., 1975; Kakiuchi et al., 1970). cAMP and calcium nearly always act together in mediating cellular response [cf. review by Rasmussen 1980)]. Examples of dual control are found in phosphorylase kinase [cf. review by Malencik & Fischer (1982)] and in smooth muscle myosin light chain kinase (Conti & Adelstein, 1981), strictly calmodulin-dependent enzymes with activities stimulated and inhibited, respectively, after phosphorylation by the cAMP-dependent protein kinase. Furthermore, in vitro phosphorylation catalyzed by protein kinase diminishes the ability of several model calmodulin-binding proteins—troponin I, the myelin basic protein, and histone H2A—to associate with calmodulin (Malencik et al., 1982a).

Model peptide studies have identified specific amino acid residues that function as determinants in the recognition and phosphorylation of seryl and threonyl residues by the cAMP-dependent protein kinase [cf. reviews by Carlson et al. (1979) and Casnellie & Krebs (1984)]. Basic residues, in particular arginine, preceding the affected serine or threonine are apparently essential. A minimal structure of -Arg-Arg-X-Ser-X- occurs in many physiologically significant protein kinase substrates [cf. review by Krebs & Beavo (1979)]. Observations on 50 or more small calmodulin-binding peptides indicated that strongly basic amino acid residues also function in the calcium-dependent binding of peptides and proteins by calmodulin. The most effective known calmodulin-binding peptides range upward in size from 9 or 10 amino acid residues and share common structural features: clusters of two or more basic amino acid residues, associated hydrophobic sequences, a low incidence of glutamyl residues, and a general propensity for either an α-helical or "random-coil" conformation [cf. review by Anderson & Malencik (1986)]. The hypothesis that similar structures occur within the calmodulin binding sites of the enzymes and other proteins that recognize calmodulin is supported by the sequence of a calmodulin-binding cyanogen bromide fragment prepared from skeletal muscle myosin light chain kinase: *KRR*WK*K*NFIAVSAAN*R*F*KK*ISSSGALM (Blumenthal et al., 1985).

The apparent similarities in specificity led to the suggestion that calmodulin and cAMP-dependent protein kinase interact with common sequences in some proteins, with modification of calmodulin binding sites being among the possible functions of protein kinase (Malencik & Anderson, 1982; Malencik et al., 1982a,b). However, differences in the specificities of the two proteins soon became evident. A synthetic peptide derived from the phosphorylation site of the β-subunit of rabbit muscle phosphorylase kinase (*RTKR*SGSVYEPL*KI*) is an excellent substrate for the cAMP-dependent protein kinase (Malencik & Anderson, 1983a) but a poor ligand for calmodulin (Malencik & Anderson, 1983b). Conversely, several high-affinity calmodulin-binding peptides—melittin (Katoh et al., 1982), mastoparan, and vasoactive intestinal peptide (VIP) (Anderson & Malencik, 1986)—inhibit protein kinase weakly

† This work was funded by the National Institutes of Health (AM 13912, AM 07441, and AM 07902), the Muscular Dystrophy Association, and the Oregon Affiliate of the American Heart Association.

* Address correspondence to this author.

‡ Department of Biochemistry and Biophysics, Oregon State University.

§ Department of Pharmacology and Howard Hughes Medical Institute, University of Washington.

‖ Department of Biochemistry, University of Washington.

0006-2960/86/0425-3502$01.50/0

or not at all. Whether there is significant partial overlap in the recognition sequences for calmodulin and protein kinase remains undetermined.

The 8000-dalton heat-stable protein inhibitor of cAMP-dependent protein kinase forms a specific high-affinity complex with the free catalytic subunit obtained upon dissociation of the holoenzyme by cAMP (Walsh et al., 1971; Ashby & Walsh, 1973). Model peptide syntheses performed in conjunction with microsequencing demonstrated that the inhibitory properties of this novel effector reside largely within its first 24 amino acid residues (Scott, 1985; Scott et al., 1985a,b; 1986). A synthetic deletion peptide representing residues 11–30 of the native inhibitor (IASG*R*TG*RR*NAIHDILVSSA) retains moderate inhibitory properties (Scott et al., 1985a). The pair of arginyl residues contained within the "pseudosubstrate" site (*RR*NAI) for protein kinase are apparently vital to the inhibitory properties of both the deletion peptide and the protein kinase inhibitor (Scott et al., 1986).

This paper describes the high-affinity binding of the aforementioned 20-residue peptide by calmodulin and its influence on the catalytic activities of two calmodulin-dependent enzymes, calcineurin and smooth muscle myosin light chain kinase. The effects of the peptide and several related analogues are compared to those of the native protein kinase inhibitor.

Materials and Methods

Materials. Turkey gizzard myosin light chain kinase was prepared essentially according to the procedure of Sobieszek and Barylko (1984). The catalytic subunit of type II cAMP-dependent protein kinase was prepared from bovine heart as described by Peters et al. (1977). The type II regulatory subunit from bovine heart was isolated according to Dills et al. (1979), with residual cAMP removed according to the procedure of Builder et al. (1979). Porcine brain calmodulin was purified according to Schreiber et al. (1981) and porcine brain calcineurin according to Klee et al. (1983). The heat-stable inhibitor of cAMP-dependent protein kinase was initially purified by the method of Demaille et al. (1977) and subjected to a final purification involving reverse-phase high-performance liquid chromatography (unpublished method). Chicken heart lactate dehydrogenase was prepared according to the procedure of Pesce et al. (1964). All proteins were homogeneous on $NaDodSO_4$[1] electrophoresis.

The peptide analogue of the 20 000-dalton smooth muscle light chain (*KKK*PQ*R*ATSNVFS-NH_2) was purchased from Peninsula Laboratories. The analogue of the phosphorylation site in the β-subunit of phosphorylase kinase (*RTKR*SGSVYEPL*KI*) was obtained from Ocean Biologics, Inc. (Edmonds, WA). The peptide analogues of the heat-stable protein kinase inhibitor were synthesized on a Beckman 990B automated solid-phase peptide synthesizer as described (Feramisco & Krebs, 1978; Scott et al., 1985a). The structure of each was confirmed by amino acid analysis and sequence determination. The phosphorylated derivative of the heat-stable protein kinase inhibitor analogue G*R*TG*RR*NSIHDIL was prepared by incubating a solution containing 80 μM of the peptide, 0.20 mM ATP, 14 μg/mL isolated catalytic subunit, 0.5 mM $MgCl_2$, 0.20 N KCl, and 50 mM Mops, pH 7.3 (25 °C). Measurements using dansylcalmodulin (see below) showed that maximum changes in binding are obtained on the addition of ~2 mol of ATP/mol of peptide, with higher proportions of ATP having no further effect. The assays described later indicate that both the threonyl and seryl residues of this peptide become phosphorylated. Since the low concentrations of magnesium and ADP/ATP present have no effect on the binding of the peptide by calmodulin (±1%), this mixture was used directly in the fluorescence titration of dansylcalmodulin.

p-Nitrophenyl phosphate, ATP (disodium salt, 99–100% from equine muscle), phosphoenolpyruvate (tricyclohexylammonium salt), NADH (disodium salt, grade III), and rabbit muscle pyruvate kinase (type VII in 50% glycerol) were obtained from Sigma Chemical Co. Reagent-grade (or best available) chemicals and distilled water passed through a Milli-Q reagent water system were used entirely.

Fluorescence Binding Measurements. Both calcium-dependent and -independent peptide binding were determined with dansylcalmodulin, prepared as described by Malencik and Anderson (1982). The changes in fluorescence obtained on the addition of varying concentrations of peptide to solutions containing fixed concentrations of dansylcalmodulin were measured with the Hitachi Perkin-Elmer MFP-2A fluorometer. The wavelengths of excitation and emission were fixed at 340 and 460 nm, respectively. Constant temperature was maintained with a circulating water bath. Mathematical analysis of the results was outlined by Malencik and Anderson (1982) and, previously, by Anderson (1974).

Catalytic Activity Measurements. The phosphatase activity of calcineurin was determined spectrophotometrically, with the artificial substrate *p*-nitrophenyl phosphate as described by Pallen and Wang (1983). The assay medium generally contained 0.97 mg/mL *p*-nitrophenyl phosphate, 1.0 mM $MnCl_2$, and 50 mM Mops, pH 7.3, 25 °C. Following the introduction of any inhibitors to be studied, the reaction was initiated by the addition of calcineurin (to 9.6 μg/mL).

To determine the basal activity of the enzyme, the absorbancy change at 405 nm (1-cm cuvette path length) was followed for 1.5 min in the Varian Model 635D spectrophotometer equipped with a Hewlett-Packard X-Y recorder. (The full-scale deflection of the recorder represented 0.05 absorbancy unit.) Calmodulin was then added and the time course of the reaction followed for another 1.5-min interval. Since we find that repeated freezing and thawing of calcineurin result in a gradual loss of activity, with the basal activity declining more quickly than that which is calmodulin-dependent, the calcineurin samples used in these experiments were frozen only once.

Smooth muscle myosin light chain kinase was assayed by the coupled, fluorometric method of Malencik and Anderson (1986). The assay medium consists of 60 μM synthetic myosin light chain kinase substrate, 0.10 mM ATP, 8.0 μM NADH, 1.0 mM PEP, 0.4 unit/mL lactate dehydrogenase, 2 units/mL pyruvate kinase, specified concentrations of calmodulin and myosin light chain kinase, 2.0 mM $MgCl_2$, 0.20 mM $CaCl_2$, and 50 mM Mops (K^+), pH 7.3, 25 °C. The rate of NADH oxidation was followed fluorometrically in the Hitachi-Perkin Elmer MPF-2A fluorometer, with fixed excitation and emission wavelengths of 348 and 455 nm. This assay is based on the coupled spectrophotometric assay for cAMP-dependent protein kinase reported by Cook et al. (1982). To show that the activities of the coupling enzymes are unaffected by the inhibitors added, control experiments were performed in which myosin light chain kinase and calmodulin were omitted from, and 0.10 mM ADP added to, the reaction mixture.

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CaM, calmodulin; PKI, heat-stable inhibitor of the cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; MLCK, myosin light chain kinase; K_d, dissociation constant; *F*, fluorescence intensity; $NaDodSO_4$, sodium dodecyl sulfate.

Table I: Interaction of Dansylcalmodulin with Peptide Analogues of Inhibitory Region of Heat-Stable Protein Inhibitor of cAMP-Dependent Protein Kinase

peptide no.	sequence[c]	[KCl] (M)	[$CaCl_2$] (mM)	[EDTA] (mM)	K_d (μM)[a]
1	IASG*RTG**RR*NAIHDILVSSA	0.20	1.0	0	~0.07, 0.09[b]
		0.20	0	0.20	8.8
2	YAFIASG*RTG**RR*NAIHDIL	0.20	1.0	0	5.8
		0	1.0	0	0.17
		0	0	0.20	6.9
3	G*RTG**RR*NSIHDIL	0.20	1.0	0	7.0
		0.20	1.0	0	63 (phosphorylated)
4	*RR*NAIHDILVSSA	0.20	1.0	0	sl inter[d]
5	IAAG*RTG**RR*NAIHE	0.20	1.0	0	sl inter[d]
6	IAAG*RTG**RR*NUIHDILVSSA	0.20	1.0	0	25

[a] Reproducibility was generally ±5–10%. Conditions: 50 mM Mops, pH 7.3 (25 °C). [b] Determined in competition experiment with smooth muscle myosin light chain kinase, using unlabeled calmodulin. [c] U = α-aminobutyrate. [d] Slight interaction.

Two different assays for cAMP-dependent protein kinase were used. The first was a coupled fluorometric assay similar to that just described for smooth muscle myosin light chain kinase. The differences between the present assay and that just described are as follows: (1) Calcium is omitted[2] and 1 mM EGTA added (except when effects of calmodulin etc. are under scrutiny). (2) KCl at 0.10 M is added. (3) Concentrations of 10 μM of the phosphorylase kinase analogue are used in place of the myosin light chain or its synthetic analogue. Under these conditions, total oxidation of NADH occurs, giving a time course that is linear for most of the reaction. However, the use of NADH concentrations in excess of the peptide gives additional information—the background adenosinetriphosphatase activity and the stoichiometry of the phosphorylation reaction. When 20 μM NADH is used,[3] 9.7 μM of NADH are rapidly oxidized [12.1 μmol min^{-1} (mg of "C")$^{-1}$] and the level of residual adenosinetriphosphatase activity is 0.44 μmol min^{-1} (mg of "C")$^{-1}$. Since there is one to one correspondence between ADP production and NADH oxidation, the results are consistent with the incorporation of 1 mol of P/mol of phosphorylase kinase analogue. A similar experiment with the phosphorylatable derivative (Scott et al., 1986) of the heat-stable protein kinase inhibitor (G*RTG**RR*NSIHDIL), using 5.36 μM peptide and 20 μM NADH, showed rapid oxidation of 10 μM NADH with the residual reaction occurring at 4.4% of the initial rate. The ratio of 1.87 NADH/mol of peptide indicates that both the serine and threonine residues of the peptide become phosphorylated.

The second assay, which is more direct, was used for the inhibition experiments. The changes in the intrinsic fluorescence of the phosphorylase kinase peptide analogue were monitored with excitation and emission wavelengths of 280 and 305 nm, following the original assay described by Malencik and Anderson (1983a). The SLM-Aminco SPF-500C spectrofluorometer was used in this study. After adjusting both slit widths to 5 nm and the emission and reference phototube voltages to 710 and 285 V, respectively, we used the instrument's offset adjustment to obtain a full-scale deflection for the course of the reaction. The reaction mixture used contained 10 μM peptide, 0.10 mM ATP, 0.10 N KCl, 2.0 mM $MgCl_2$, 0.20 mM $CaCl_2$, and 5.0 mM Mops, pH 7.3 (25 °C).

Results

Association of Peptide Analogues with Dansylcalmodulin. Dansylcalmodulin is an unusually responsive covalent conjugate prepared by labeling calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride. The fluorescence emission maximum and quantum yield of dansylcalmodulin change dramatically on the binding of both calcium and proteins or peptides [Malencik et al., 1981; Malencik & Anderson, 1982; review by Anderson & Malencik (1986)]. Figure 1 illustrates the fluorescence increase measured at 460 nm when dansylcalmodulin is titrated with varying concentrations of the first five peptide analogues listed in Table I. The results are plotted on a logarithmic scale since the peptides vary widely in their affinities for calmodulin. The fractional degree of saturation (ϕ) of dansylcalmodulin with the individual peptides is related to the fluorescence enhancement:

$$\phi = (F/F_0 - 1)/(F_\infty/F_0 - 1)$$

The values of F_∞, the fluorescence of totally bound dansylcalmodulin, are determined through extrapolation of the changes in fluorescence to infinite peptide concentration. Double-reciprocal plots of the changes in fluorescence $(F/F_0 - 1)^{-1}$ vs. the calculated concentrations of free peptide, $([\text{peptide}]_{total} - \phi[\text{CaM}]_{total})^{-1}$, are generally linear—allowing

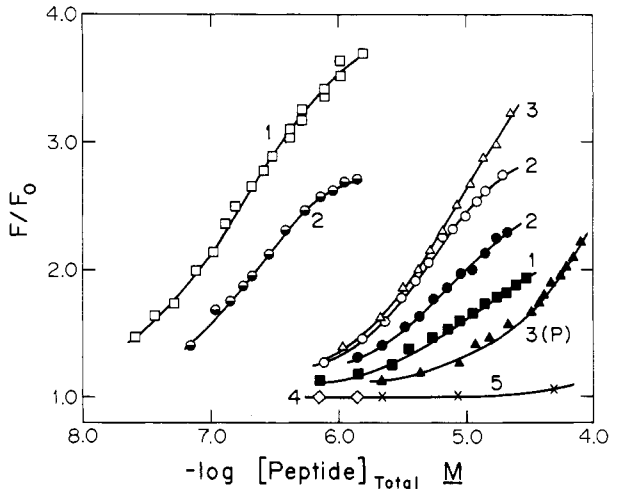

FIGURE 1: Binding of five peptide analogues of the heat-stable cAMP-dependent protein kinase inhibitor by dansylcalmodulin. The relative fluorescence intensities (F/F_0) were measured as a function of varying peptide concentrations. (F_0 is the fluorescence of dansyl-CaM in the absence of added peptide.) General conditions: 50 mM Mops, pH 7.3 (25 °C). Refer to Table I for sequences. Peptide 1: 0.08 μM dansyl-CaM, 0.20 M KCl, and 1.0 mM $CaCl_2$ (□); 0.80 μM dansyl-CaM, 0.20 M KCl, and 0.20 EDTA (■). Peptide 2: 0.80 μM dansyl-CaM, 0.20 M KCl, and 1.0 mM $CaCl_2$ (○); 0.08 μM dansyl-CaM, 0 KCl, and 1.0 mM $CaCl_2$ (⊖); 0.08 μM dansyl-CaM, 0 KCl, and 0.20 mM EDTA (●). Peptide 3: 0.80 μM dansyl-CaM, 0.20 M KCl, and 1.0 mM $CaCl_2$ [(△) before and (▲) after peptide phosphorylation]. Peptides 4 (◇) and 5 (×): 0.80 μM dansyl-CaM, 0.20 M KCl, and 1.0 mM $CaCl_2$.

[2] Calcium is moderately inhibitory to the cAMP-dependent protein kinase.

[3] The fluorescence intensities of NADH solutions are essentially proportional to concentration, with 2.7% deviation from linearity, through the range of 0–8 μM. Standard curves of intensity vs. [NADH] are used in the interpretation of assays performed at concentrations above 10 μM.

determination of the dissociation constants (K_d) and verification of the values of F_∞/F_0. Since values of $[CaM]_{total} \leq K_d$ minimize the propagation of experimental error, the calmodulin concentration was reduced to 0.08 μM for the two cases in which $K_d \ll 1$ μM.

The 20-residue analogue (refer to peptide 1 in Table I) of the inhibitory region of the heat-stable protein kinase inhibitor has a relatively high affinity for calmodulin, comparable to that previously found with VIP (Malencik & Anderson, 1983b). The dissociation constant determined from the midrange of the saturation curve,[4] ~0.07 μM, approaches the lower limit (~0.05 μM) for precise values of K_d obtainable with dansylcalmodulin. Competitive displacement methods, such as that based on the calmodulin-dependent binding of 9-anthroylcholine by smooth muscle myosin light chain kinase [Malencik et al., 1982a; review by Anderson & Malencik (1985)], are used when K_d is in the nanomolar range. Table I also contains a value for K_d (0.09 ± 0.03 μM) determined by the latter method (experimental data not shown). These experiments were both performed under standard conditions (0.20 M KCl, 50 mM Mops, 1.0 mM $CaCl_2$, pH 7.3, 25 °C) routinely used to characterize peptide binding by calmodulin. The calcium dependence of the 20-residue peptide–dansylcalmodulin interaction was established in separate measurements with buffer solutions containing added EDTA (0.2 mM) and no calcium. Under these conditions, the value of K_d increases to 8.8 μM.

The C-terminal -Val-Ser-Ser-Ala sequence of the 20-residue peptide is apparently strongly stabilizing. The second peptide, which lacks these residues but contains an N-terminal extension, exhibits moderate affinity for calmodulin. Under the standard conditions, K_d = 5.8 μM. The value of K_d decreases to 0.17 μM, with continuing strong calcium dependence, when KCl is omitted from the buffer. Comparison of the amino acid sequence and binding properties of the third analogue to those of the other five suggests that it approaches a minimal structure still compatible with efficient binding (K_d < 10 μM) by calmodulin. This peptide also contains an amino acid replacement, Ala → Ser, which allows it to be phosphorylated by the cAMP-dependent protein kinase (Scott et al., 1986). The effect of phosphorylation on the binding of this peptide by dansylcalmodulin, with K_d increasing from 7.0 to 63 μM, is larger than the changes previously found with two other phosphorylatable peptides (Malencik et al., 1982b; Malencik & Anderson, 1983b). This probably relates to the occurrence of *two* phosphorylatable sites within the present derivative. The fourth and fifth peptides show the effects of further N- and C-terminal deletions, respectively. The replacement Ala → α-aminobutyrate made in the sixth peptide has a dramatic effect on calmodulin binding, which even exceeds that observed by Scott et al. (1986) with protein kinase.

The avid binding of the 20-residue analogue by calmodulin is a unique property not shared with the native heat-stable cAMP-dependent protein kinase inhibitor. Solutions containing 1.0 μM dansylcalmodulin and 1.4 μM of the native inhibitor show incomplete interaction under conditions where the duodecapeptide would nearly saturate calmodulin. Although the quantity of native inhibitor available precludes precise determination of K_d, estimations suggest a value of ~3 μM for the calmodulin–inhibitor complex under the standard conditions of these measurements (0.2 M KCl, 50 mM Mops, 1 mM $CaCl_2$). Parallel experiments with both the type II regulatory subunit and the isolated catalytic subunit revealed no interactions with dansylcalmodulin occurring in the micromolar concentration range. The enzyme inhibition experiments presented in the next section deal further with the differences between the duodecapeptide analogue and the native protein kinase inhibitor.

[4] The extremes of the titration show slight heterogeneity, which is intrinsic to dansylcalmodulin.

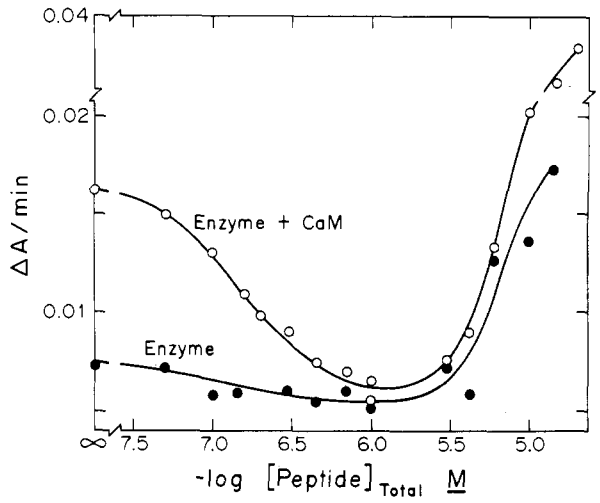

FIGURE 2: Inhibition of porcine brain calcineurin by the 20-residue (peptide 1) analogue of the heat-stable inhibitor of cAMP-dependent protein kinase. The rate of absorbancy change at 405 nm is plotted as a function of the total peptide concentration. Conditions: 0.97 mg/mL *p*-nitrophenyl phosphate, 1.0 mM $MnCl_2$, and 50 mM Mops, pH 7.3 (25 °C). (○) 0.12 μM enzyme + 0.10 μM calmodulin; (●) 0.12 μM enzyme + 0 calmodulin.

Enzyme Inhibition. Spectrophotometric assays using the synthetic substrate *p*-nitrophenyl phosphate showed that several metal ions in addition to calcium stimulate the calmodulin-dependent phosphatase activity of calcineurin. Of these, Mn^{2+} is especially useful for quantitative activity determinations. It gives time courses that are nearly linear for several minutes whereas those obtained with Ca^{2+} are markedly nonlinear (Pallen & Wang, 1983). Figure 2 illustrates the effect of varying concentrations of the duodecapeptide on both the calmodulin-dependent and the calmodulin-independent phosphatase activities of calcineurin determined with 0.97 mg/mL *p*-nitrophenyl phosphate, 1.0 mM $MnCl_2$, and 50 mM Mops, pH 7.3 (25 °C). Low concentrations of the peptide (0.025–1.0 μM) have minimal effect on the basal activity of calcineurin. However, the potent inhibition of the calmodulin-dependent activity—with 50% inhibition occurring at 0.13 μM peptide—indicates that the affinity of the 20-residue peptide for calmodulin rivals that of the enzyme under the conditions of the experiment. Unexpectedly, higher concentrations of the 20-residue peptide (>3 μM) proved stimulatory to the basal activity of calcineurin. Repetition of key measurements in an assay system containing 1.0 mM $CaCl_2$ and no $MnCl_2$ confirmed the dual effect of inhibition of calmodulin-dependent activity at low peptide concentrations and activation of basal activity at high concentrations. We find that melittin, whose binding by calmodulin was first reported by Comte et al. (1983), also inhibits the calmodulin-dependent activity of calcineurin (with 50% inhibition found at 0.11 μM melittin) but has little effect on the base-line activity at tested concentrations up to 10 μM.

The associations of calcineurin and calmodulin with the native cAMP-dependent protein kinase inhibitor were examined in two types of experiment. The results in Table II indicate that the presence of the inhibitor at a concentration of 0.14 μM depresses both the basal and the calmodulin-dependent phosphatase activity of calcineurin. Note that these

Table II: Effects of PKI on Catalytic Activity of Calcineurin

[Mn^{2+}] (mM)	additions			reaction rate (ΔA/min)[a]
	[Ca^{2+}] (mM)	[CaM] (μM)	[PKI] (μM)	
1.0	0	0	0	0.0065
1.0	0	0.10	0	0.015
1.0	0	0	0.14	0.0028
1.0	0	0.10	0.14	0.0086
0	1.0	0	0	0.0038
0	1.0	0.10	0	0.010
0	1.0	0	0.14	0.0028
0	1.0	0.10	0.14	0.0046

[a] Reproducibility was ±6% in the presence of CaM and ±10–15% in absence of CaM. Conditions: 9.6 μg/mL calcineurin, 0.97 mg/mL *p*-nitrophenyl phosphate, and 50 mM Mops, pH 7.3, 25 °C. Change in absorbance was measured at 405 nm.

Table III: Effects of Calmodulin and Calcineurin on Inhibition of the cAMP-Dependent Protein Kinase by PKI

Additions			reaction rate (μM/min)[a]
[CaM] (μM)	[PKI] (nM)	[calcineurin] (μM)	
0	0	0	1.8
0	0	0.13	2.0
0	17	0	0.04
0.5	0	0	1.8
0.5	17	0	0.05
0.5	17	0.13	0.13

[a] Reproducibility at the optimum rate was ±0.15 μM/min. Conditions: 0.25 μg/mL "C", 10 μM RTKRSGSVYEPLKI, 0.10 mM ATP, 2.0 mM $MgCl_2$, 0.20 mM $CaCl_2$, 0.10 M KCl, and 5.0 mM Mops, pH 7.3 (25 °C).

assays were performed both with Mn^{2+} and with Ca^{2+}. (In the latter case, the basal reaction rate was measured 1 min after initiation of the reaction with added enzyme; the rate of the calmodulin-dependent reaction was determined 4 min after the initial addition of enzyme and 1 min after the addition of calmodulin.)

To determine whether either calcineurin or calmodulin competes effectively with the isolated catalytic subunit of protein kinase in the binding of the native heat-stable inhibitor, we applied the continuous fluorometric assay for cAMP-dependent protein kinase described by Malencik and Anderson (1983a). Phosphorylation of the synthetic peptide—*RTKR*SGSVYEPL*KI*—derived from the phosphorylation site of the β-subunit of phosphorylase kinase occurs almost entirely at a single residue (probably serine at position 7). The reaction is accompanied by a 36% decrease in the intrinsic fluorescence of the peptide, an effect predicted from the collisional quenching of tyrosine by the nearby phosphate ester moiety. Fluorometric assays based on this change give linear time courses at the peptide concentration customarily used (10 μM) and respond immediately to additions of the protein kinase inhibitor. The experiments in Table III were performed under the original assay conditions with two modifications: deletion of EGTA and addition of 0.20 mM $CaCl_2$.[2] The inclusion of calmodulin and/or calcineurin in the assay has negligible effects on the activity of the isolated catalytic subunit alone. The nearly complete inhibition obtained with the heat-stable protein kinase inhibitor is also largely unaffected (within the margin of experimental error) by either calmodulin or calcineurin—even at the excess concentrations used. These results are independent of the order of mixing of the proteins concerned.

Unlike calcineurin, smooth muscle myosin light chain kinase is a rather strictly calmodulin-dependent enzyme [cf. review by Perry et al. (1984)]. The catalytic activity determinations summarized in Table IV show that the 20-residue analogue—but not the native protein kinase inhibitor—is a potent antagonist of myosin light chain kinase. Note that this assay uses the synthetic smooth muscle myosin light chain kinase substrate developed by Kemp et al. (1983) in place of the 20000-dalton myosin light chain. The adoption of the synthetic substrate is necessary since many calmodulin binding proteins and peptides associate appreciably with the myosin light chain at the concentrations used in activity determinations [cf. review by Anderson & Malencik (1985)]. (Solutions containing 10 μM each of the 20000-dalton myosin light chain and the 20-residue peptide are in fact conspicuously turbid.) The nearly complete inhibition obtained with 1 μM concentrations of the analogue is quickly reversed (90% recovery) on the addition of excess calmodulin (4 μM). The inhibition is just moderately diminished by the inclusion of 0.10 M KCl in the assay medium, with 53% inhibition determined at a peptide concentration of 0.10 μM.

Table IV: Effects of PKI and Inhibitory 20-Residue Analogue on Catalytic Activity of Smooth Muscle Myosin Light Chain Kinase

additions			reaction rate (μM/min)[a]
[peptide] (μM)	[PKI] (μM)	[CaM] (μM)	
0	0	0.010	1.27
0.054	0	0.010	0.69
0.10	0	0.010	0.36
1.0	0	0.010	0.05
1.0	0	4.0	1.14
0	0.14	0.010	1.30

[a] Reproducibility was ±0.08 μM/min. Conditions: 10.0 nM MLCK, 60 μM synthetic light chain analogue, 0.10 mM ATP, 2.0 mM $MgCl_2$, 0.20 mM $CaCl_2$, 50 mM Mops (pH 7.3, 25 °C), plus coupled assay system described in the text.

Discussion

The peptide analogues of the heat-stable inhibitor of the cAMP-dependent protein kinase have provided the first opportunity to directly compare the binding specificities of calmodulin and protein kinase. In both cases, the most strongly bound peptides contain the -Arg-Thr-Gly-Arg-Arg- sequence. However, the amino acid sequences adjoining the N- and C-terminal ends of this basic center apparently contribute differently to the peptide complexes formed by the two proteins. Of the peptides studied here, number two is the most effective inhibitor of the cAMP-dependent protein kinase (K_I = 0.22 μM), with peptides 1 and 5 being next most effective (K_I = 0.8 μM for each). The third peptide is a substrate of protein kinase (K_m = 220 μM) while the fourth is a weak inhibitor (K_I = 1500 μM) (Scott, 1985; Scott et al., 1985a, 1986). Calmodulin, in contrast, does not interact appreciably with the fourth and fifth peptides. It exhibits moderate binding of the second and third peptide analogues and avid binding of the first (refer to Table I). The N-terminal YAFIAS-sequence is evidently strongly stabilizing in the peptide complexes formed with the cAMP-dependent protein kinase but not with calmodulin. On the other hand, the C-terminal -ILVSSA sequence is necessary for efficient binding by calmodulin but not by protein kinase (Scott et al., 1986). The substitution of an α-aminobutyrate residue for alanine in peptide 6 markedly diminishes its binding by calmodulin. Similar results obtained with protein kinase were explained by steric effects (Scott et al., 1986). However, additional factors may be involved in the case of calmodulin. Normal amino acid residues such as leucine and isoleucine generally have above average rates of occurrence in calmodulin-binding

peptides (Anderson & Malencik, 1986).

Application of structure prediction rules [cf. review by Chou & Fasman (1978)] indicates that the last ten amino acid residues of the 20-residue peptide (number one) may form an amphipathic α-helix while the residues associated with the basic center (positions 2–10) are likely to occur within β-turns. Similar mixed secondary structures have been predicted for a number of calmodulin-binding peptides [cf. review by Anderson & Malencik (1986)]. The failure of the native heat-stable protein kinase inhibitor to bind calmodulin as effectively as the 20-residue peptide suggests the inaccessibility of critical portions of the sequence that are available in the synthetic analogue. Possibly, the hydrophobic amino acid side chains occurring in the amphipathic α-helix are located in the interior of the native inhibitor molecule.

The sensitivity of the complexes to changes in ionic strength [see also Malencik & Anderson (1984)] and the effect of peptide phosphorylation on binding are consistent with the predicted stabilizing role of the positively charged amino acid side chains (Malencik & Anderson, 1982). Although changes in calmodulin binding accompanying the phosphorylation of several peptides and model proteins now have been reported (Malencik et al., 1982a,b; Malencik & Anderson, 1983; Villar-Palasi et al., 1983), none is as large as that obtained when smooth muscle myosin light chain kinase is phosphorylated in vitro by the cAMP-dependent protein kinase (Conti & Adelstein, 1981; Malencik et al., 1982a). The 20–100-fold decrease in binding occurring in the latter case may reflect conformational changes, although none have been detected, rather than immediate covalent modification of the calmodulin binding site.

The effects of the 20-residue peptide and the native heat-stable protein kinase inhibitor on the catalytic activity of smooth muscle myosin light chain kinase are in accord with the results of the binding experiments performed with dansylcalmodulin. However, the peptide has a dual effect on the activity of calcineurin—with inhibition of calmodulin-dependent phosphatase activity noted at low peptide concentrations and activation of calmodulin-independent activity prevailing at high concentrations. The native protein kinase inhibitor, on the other hand, depresses both the calmodulin-dependent and -independent activities of calcineurin. Apparently, both the peptide and the protein kinase inhibitor interact with calcineurin in the absence of calmodulin. However, the failure of excess concentrations of calcineurin or calmodulin to protect the isolated catalytic subunit of cAMP-dependent protein kinase from inhibition by the native heat-stable inhibitor indicates that the primary interaction of the inhibitor is with protein kinase.

Registry No. Peptide 1, 97729-58-3; peptide 2, 102047-19-8; peptide 3, 102047-20-1; peptide 4, 97729-60-7; peptide 5, 102047-21-2; peptide 6, 102047-22-3; myosin light chain kinase, 51845-53-5; protein kinase, 9026-43-1.

REFERENCES

Anderson, S. R. (1974) in *Experimental Techniques in Biochemistry* (Brewer, J. M., Pesce, A. J., & Ashworth, R. B., Eds.) pp 248–261, Prentice-Hall, Englewood Cliffs, NJ.

Anderson, S. R., & Malencik, D. A. (1986) *Calcium Cell Funct. 6*, 1.

Ashby, C. D., & Walsh, D. A. (1973) *J. Biol. Chem. 248*, 1255.

Blumenthal, D, K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Brostrom, C. O., Huang, Y. C., Breckenridge, B. M., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64.

Builder, S. E., Beavo, J. A., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 3514.

Carlson, G. M., Bechtel, P. J., & Graves, D. J. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 41.

Casnellie, J. E., & Krebs, E. G. (1984) *Adv. Enzyme Regul. 22*, 501.

Cheung, W. Y. (1971) *J. Biol. Chem. 246*, 2859.

Cheung, W. Y. (1981) *Calcium Cell Funct. 1*, 1.

Cheung, W. Y., Gradham, L. S., Lynch, T. J., Lin, Y. M., & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun. 66*, 1055.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251.

Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269.

Conti, M. A., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 3178.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R. (1982) *Biochemistry 21*, 5794.

Corbin, J. D., & Reimann, E. G. (1974) *Methods Enzymol. 38*, 287.

Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080.

Dills, W. L., Goodwin, C. D., Lincoln, T. M., Beavo, J. A., Bechted, P. J., Corbin, J. D., & Krebs, E. G. (1979) *Adv. Cyclic Nucleotide Res. 10*, 199.

Feramisco, J. R., Glass, D. B., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 4240.

Kakiuchi, S., Yamazaki, R., & Nabajima, H. (1970) *Proc. Jpn. Acad. 46*, 587.

Katoh, N., Raynor, R. L., Wise, B. C., Schatzman, R. C., Turner, R. C., Helfman, D. M., Fain, J. N., & Kuo, J.-F. (1982) *Biochem. J. 202*, 217.

Kemp, B. E., Pearson, R. B., & House, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7472.

Klee, C. B., Krinks, M. H., Manalan, A. S., Cohen, P., & Stewart, A. A. (1983) *Methods Enzymol. 102*, 227.

Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923.

Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480.

Malencik, D. A., & Fischer, E. H. (1982) *Calcium Cell Funct. 3*, 161.

Malencik, D. A., & Anderson, S. R. (1983a) *Anal. Biochem. 132*, 34.

Malencik, D. A., & Anderson, S. R. (1983b) *Biochemistry 22*, 1995.

Malencik, D. A., & Anderson, S. R. (1984) *Biochemistry 23*, 2420.

Malencik, D. A., & Anderson, S. R. (1986) *Biochemistry 25*, 709.

Malencik, D. A., Anderson, S. R., Shalitin, Y., & Schimerlik, M. I. (1981) *Biochem. Biophys. Res. Commun. 101*, 390.

Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. (1982a) *Biochemistry 21*, 4031.

Malencik, D. A., Huang, T.-S., & Anderson, S. R. (1982b) *Biochem. Biophys. Res. Commun. 108*, 266.

Pallen, C. J., & Wang, J. H. (1983) *J. Biol. Chem. 258*, 8550.

Perry, S. V., Cole, H. A., Hudlicka, O., Patchell, V. B., & Westwood, S. A. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 3015.

Pesce, A., McKay, R., Stolzenbach, F., Cahn, R. D., & Kaplan, N. O. (1964) *J. Biol. Chem. 239*, 1753.

Peters, K. A., Damille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.
Rasmussen, H. (1980) *Ann. N.Y. Acad. Sci. 356*, 346.
Schreiber, W. E., Sasagawa, T., Titani, K., Wade, R. D., Malencik, D. A., & Fischer, E. H. (1981) *Biochemistry 20*, 5239.
Scott, J. D. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 69*, 703.
Scott, J. D., Fischer, E. H., Demaille, J. G., & Krebs, E. G. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4379.
Scott, J. D., Fischer, E. H., Takio, K., Demaille, J. G., & Krebs, E. G. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5732.
Scott, J. D., Glaccum, M. B., Fischer, E. H., & Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1613.
Sobieszek, A., & Barylko, B. (1984) in *Smooth Muscle Contraction* (Stephens, N. L., Ed.) p 283, Dekker, New York.
Villar-Palasi, C., Oshiro, D., & Kretsinger, R. H. (1983) *Biochim. Biophys. Acta 757*, 40.
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1977.

Isolation and Identification of Cysteinyl Peptide Labeled by 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate in the Reduced Diphosphopyridine Nucleotide Inhibitory Site of Glutamate Dehydrogenase†

Surendra P. Batra and Roberta F. Colman*
Department of Chemistry, University of Delaware, Newark, Delaware 19716
Received November 13, 1985; Revised Manuscript Received February 19, 1986

ABSTRACT: 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TADP) has been shown to react at the reduced diphosphopyridine nucleotide (DPNH) inhibitory site of bovine liver glutamate dehydrogenase with incorporation of 1 mol of reagent/mol of enzyme subunit [Batra, S. P., & Colman, R. F. (1984) *Biochemistry 23*, 4940–4946]. The modified enzyme had lost one of the six free sulfhydryl groups per enzyme subunit as detected by 5,5′-dithiobis(2-nitrobenzoate). In the unmodified enzyme digested with trypsin, six cysteinyl peptides labeled with [^{14}C]iodoacetic acid were detected by high-performance liquid chromatography (HPLC), whereas only five were observed in the 6-BDB-TADP-modified enzyme. A cysteinyl peptide has been isolated from modified enzyme digested with trypsin and chymotrypsin. Purification of the nucleotidyl peptide was accomplished by chromatography on phenyl boronate–agarose, followed by gel filtration on Sephadex G-25 and Bio-Gel P-4 in 50 mM ammonium bicarbonate, pH 8.0. The modified peptides were finally purified by HPLC on a C_{18} column using 0.1% trifluoroacetic acid with an acetonitrile gradient. By comparison of the amino acid composition and N-terminal residue of the isolated peptide with the known amino acid sequence of the enzyme, the peptide in the DPNH inhibitory site labeled by 6-BDB-TADP has been identified as the 19-membered fragment from Glu-311 to Lys-329. A unique residue, Cys-319, was identified as the reactive amino acid within the DPNH inhibitory site.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme whose activity is modulated by purine nucleotides: adenosine 5′-diphosphate (ADP)[1] activates, GTP inhibits, and relatively high concentrations of DPNH inhibit by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972; Eisenberg et al., 1976). The enzyme is composed of six identical polypeptide chains, each of which has been reported to have a site for ADP, two sites for GTP, and two sites for DPNH (Sund et al., 1975; Pal & Colman, 1979). The amino acid sequence of glutamate dehydrogenase has been established (Julliard & Smith, 1979). For the identification of essential amino acid residues in the regulatory sites of glutamate dehydrogenase, the reactions of several purine nucleoside affinity labels with the enzyme have been studied (Colman, 1983). For example, the affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) has been reported to react specifically, with 0.5–1.0 mol of reagent incorporated per enzyme subunit, at the DPNH inhibitory site of glutamate dehydrogenase (Pal et al., 1975; Saradambal et al., 1981). Schmidt and Colman (1984) demonstrated that the residues in the DPNH inhibitory site modified by 5′-FSBA are Lys-420 and Tyr-190.

We have recently shown that the new reactive adenine nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TADP) also acts as an affinity label of the DPNH inhibitory site of glutamate dehydrogenase with incorporation of about 1 mol of reagent per peptide chain (Batra & Colman, 1984). The modified enzyme has been shown to be catalytically active, and many

† This work was supported by U.S. Public Health Service Grant GM-21200.

[1] Abbreviations: 6-BDB-TADP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate; ADP, adenosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; DPNH, reduced diphosphopyridine nucleotide; CM, carboxymethylated; DTNB, 5,5′-dithiobis(2-nitrobenzoate); PBA, phenyl boronate–agarose; Tris, tris(hydroxymethyl)aminomethane; 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-3508$01.50/0